

FINAL REPORT

Development of Biomarkers for Assessing In Situ RDX Biodegradation Potential

SERDP Project ER-1606

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14. ABSTRACT The objective was to identify the microorganisms and genes responsible for the biodegradation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in mixed microbial communities using stable isotope probing, quantitative PCR and high throughput sequencing. The research targeted RDX biodegradation in mixed community samples, as these microbial communities are more representative of those at contaminated sites. The research involved four major projects, the first of which was a literature review. The second task examined the RDX degrading communities in four different soil slurries. The third task examined the microorganisms involved in RDX biodegradation from surface soils from a detonation area at a Navy Base. The fourth task investigated the microorganisms and functional genes (xenA, xenB and xplA) linked to RDX biodegradation at two Navy sites. Several key microorganisms were associated with RDX removal in these mixed communities. These phylogenetic and functional biomarkers will be useful to determine the biological potential for RDX degradation across sites.					
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ACRONYM LIST

SERDP	Strategic Environmental Research and Development Program
BD	Buoyant density
CsCl	Cesium chloride
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
HMX	high melting explosive, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	High performance liquid chromatography
LB	Luria Bertani broth
MXN	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
SIP	Stable isotope probing
RDX	Royal demolition explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA	Ribonucleic acid
TNX	Hexahydro-1,3,5-trinitroso -1,3,5-triazine
TRFLP	Terminal restriction fragment length polymorphism

KEYWORDS

RDX, *xenA*, *xenB*, *xplA*, stable isotope probing, quantitative PCR, high throughput sequencing, *Pseudomonadeae*, *Comamonas*, *Brevundimonas*, *Bacillaceae* 1, *Rhodococcus*, *Arthrobacter*, *Sporolactobacillus*

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ABSTRACT

Objective

The objective was to identify the microorganisms and genes responsible for the biodegradation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in mixed culture samples through the application of stable isotope probing (SIP), quantitative PCR (qPCR) and high throughput sequencing (Illumina MiSeq). The work was funded under the SERDP SON ERSON-08-02 entitled “Improved understanding of the biological degradation of nitroamines in the environment”. The research specifically targeted RDX biodegradation in mixed community samples, as these microbial communities are more representative of those at contaminated sites.

Technical Approach

The research involved four major projects, the first of which was a literature review to identify the dominant microorganisms linked to RDX biodegradation in pure and mixed culture samples. The second task examined the RDX degrading communities in four different soil slurries. High throughput sequencing was used to determine which phylotypes experienced an increase in relative abundance following RDX degradation. For this, total genomic DNA was sequenced from the 1) initial soils, 2) soil slurry microcosms following RDX degradation and 3) control soil slurry microcosms without RDX addition.

The third task examined the microorganisms involved in RDX biodegradation from surface soils from a detonation area at a Navy Base. Microbial communities were compared between the initial sample, samples following RDX degradation and controls not amended with RDX to determine which phylotypes increased in abundance following RDX degradation. The effect of glucose on these communities was also examined. In addition, stable isotope probing (SIP) using labeled ($^{13}\text{C}_3$, $^{15}\text{N}_3$ – ring) RDX was performed.

The fourth task investigated the microorganisms and functional genes (*xenA*, *xenB* and *xplA*) linked to RDX biodegradation in microcosms composed of sediment or groundwater from two Navy sites using qPCR and high throughput sequencing. For this, experiments included sediment samples from three depths (5 to 30 feet) from two wells located in one Navy site. In addition, the groundwater upstream and downstream of an emulsified oil biobarrier was examined from another Navy site. Further, for the groundwater experiments, the effect of glucose addition was explored.

Results

The literature review indicated that from the phyla with known RDX degrading isolates, *Firmicutes* and *Proteobacteria* (particularly *Gammaproteobacteria*) were the most dominant microorganisms in many contaminated site derived samples.

The most notable result from the second task was the increase in *Brevundimonas* and/or unclassified *Bacillaceae* 1 in the four soils studied during RDX degradation. Although isolates of the family *Bacillaceae* 1 have previously been linked to RDX degradation, isolates of the genus *Brevundimonas* have not been previously associated with RDX degradation. Overall, the data suggest these two phylotypes have key roles in RDX degradation in these soil communities.

The third task, focused on surface soils from a denotation area, revealed several phylotypes were more enriched during RDX degradation. This trend was strong for unclassified *Pseudomonadaceae*, *Comamonas* and *Acinetobacter*. *Rhodococcus*, a known RDX degrader, also increased in abundance following RDX degradation. SIP indicated that unclassified *Pseudomonadaceae* was the most abundant phylotype in the heavy fractions. In the glucose amended heavy fractions, *Comamonas* and *Anaeromyxobacter* were also present.

As part of the fourth task, for the sediment experiments, the most enriched phylotypes during RDX degradation varied over time, by depth and well locations. However, several trends were noted, including the enrichment of *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and *Sporolactobacillus* in the sediment microcosms. For the groundwater experiments, *Pseudomonas*, unclassified *Rhodocyclaceae*, *Sphingomonas* and *Rhodococcus* were also highly abundant during RDX degradation. Both *xplA* and *xenA* increased during RDX degradation for many treatments. In a limited number of microcosms, *xenB* gene copies increased. Phylotype data were correlated with functional gene data to highlight potentially important biomarkers for RDX biodegradation at these two Navy sites.

Benefits

Several key microorganisms were associated with RDX removal in these mixed communities. For example, *Pseudomonadaceae* were particularly dominant in many RDX degrading microcosms. Both *xplA* and *xenA* increased during RDX degradation for many treatments and in a limited number of microcosms, *xenB* gene copies increased. These phylogenetic and functional biomarkers can be used to determine the biological potential for RDX degradation across sites.

1.0 PROJECT OBJECTIVES AND BACKGROUND

1.1. Objectives

The overall objective of this work was to identify the microorganisms and genes responsible for the biodegradation of RDX in mixed culture samples through the application of the molecular methods stable isotope probing (SIP), quantitative PCR (qPCR) and high throughput sequencing. This work was funded under the SERDP Statement of Need ERSON-08-02 entitled “Improved understanding of the biological degradation of nitroamines in the environment”. The project focused on the development and application of methods required for an increased understanding of the biological degradation of nitroamines. The work specifically targeted RDX biodegradation in mixed community, complex samples as these are more representative of the transformation that would occur *in situ* at contaminated sites.

1.2. Background

The project addresses the problem of the widespread contamination of DoD sites with explosives. Such contamination has been associated with manufacturing and load-assemble-package (LAP) processes performed during or after World War II and the Korean Conflict. Remediation of many of these sites has been initiated since the early 1980s, however many still have groundwater contaminated with nitroaromatics. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is the most problematic of these because of its high frequency of use, high solubility, recalcitrance to abiotic and biotic processes and toxicity. The U.S. Environmental Protection Agency (EPA) has established health advisory (HA) levels for RDX in drinking water (2 µg/L). The health advisory indicates the potential threat that this chemical poses to humans and other organisms. Recently, attempts to remediate RDX contaminated sites have focused on biostimulation to promote RDX biodegradation. Although many RDX degrading isolates have been obtained in the laboratory, little is known about the potential of microorganisms to degrade this chemical while existing in a soil community. Additional background is provided in the following literature review (section 2.0.) and in the introduction sections (3.1., 4.1. and 5.1.) of each of the following chapters.

2.0. RDX Degrading Microbial Communities and the Prediction of Microorganisms Responsible for RDX Bioremediation

This review chapter was published (Cupples 2013), as follows:

Cupples AM. 2013. RDX degrading microbial communities and the prediction of microorganisms responsible for RDX bioremediation. *International Biodeterioration and Biodegradation* 85:260-270.

Abstract

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has caused significant soil and groundwater contamination. To remediate these sites, there is a need to determine which microorganisms are responsible for *in situ* biodegradation of RDX to enable the appropriate planning of bioremediation efforts. Here, studies are examined that have reported on the microbial communities linked with RDX biodegradation. Dominant microorganisms across samples are discussed and summarized. This information is then compared to current knowledge on RDX degrading isolates to predict which organisms may be responsible for RDX degradation in soils and groundwater. From the phyla with known RDX degrading isolates, *Firmicutes* and *Proteobacteria* (particularly *Gammaproteobacteria*) were the most dominant organisms in many contaminated site derived samples. Organisms in the phyla *Deltaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* were dominant in these studies less frequently. Notably, organisms within the class *Betaproteobacteria* were dominant in many samples and yet this class does not appear to contain any known RDX degraders. This analysis is valuable for the future development of molecular techniques to track the occurrence and abundance of RDX degraders at contaminated sites.

2.1. Introduction

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has caused widespread environmental contamination. As this chemical does not adsorb strongly to soil (Pennington and Brannon 2002; Sheremata et al. 2001) it can move to groundwater and therefore has the potential to move offsite. Currently, there are no federal drinking water standards for RDX, however, the

U.S. Environmental Protection Agency has established a health advisory level in drinking water (2 µg/L) (EPA 2011).

Bioremediation is becoming an increasingly common approach for remediating contaminated groundwater. This approach has also been applied for RDX contaminated sites, however, the microorganisms responsible for RDX degradation *in situ* have still to be fully elucidated. The aim of this review is to summarize organisms commonly found in RDX degrading mixed communities as a step towards predicting which are important for RDX degradation *in situ*. This is a timely review because although there are numerous reviews on the isolates and pathways for RDX degradation (Crocker et al. 2006; Hawari et al. 2000; Khan et al. 2012; Rylott et al. 2011b; Singh et al. 2012; Ye et al. 2004), no review exists on the mixed communities associated with RDX degradation. Such information is valuable because although isolates may be able to degrade RDX in the laboratory, the same species may not survive or be capable of RDX degradation at a contaminated site. Knowledge of the organisms linked to *in situ* RDX degradation could facilitate the prediction of whether RDX bioremediation is feasible across sites.

This review will first briefly summarize the common RDX degradation pathways and known RDX degrading functional genes. Then, the classification and source of known RDX degrading isolates will be discussed. Following this, the microbial communities associated with RDX degradation in mixed communities (soil or groundwater microcosms, site samples) will be examined. Finally, based on a review of these studies, hypotheses will be provided on which organisms are likely to be the important for RDX degradation at contaminated sites.

2.2. RDX Degrading Pathways and Functional Genes

RDX is subject to biodegradation through a number of pathways (Crocker et al. 2006; Hawari et al. 2000; Khan et al. 2012; Rylott et al. 2011b; Singh et al. 2012; Ye et al. 2004). The nitro groups are often involved in the first transformation steps, by reduction or by denitration.

The majority of research on the degradation of RDX has focused on anaerobic pathways (Khan et al. 2012; Ye et al. 2004). Under anaerobic conditions, the sequential reduction of RDX can proceed through hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-1,3,5-triazine (TNX). These products can degrade further through ring cleavage and the products generated can

be mineralized (McCormick et al. 1981). Denitration has also been reported under anaerobic conditions, involving ring cleavage and methylene dinitramine (MEDINA) formation (Zhao et al. 2002). For two strains, anaerobic denitration has been documented with both 4-nitro-2,4-diazabutanal (NDAB) and MEDINA as ring cleavage products .

Under aerobic conditions, denitration appears to be a common pathway. Denitration has been documented for many *Actinobacteria*, including, for example, *Rhodococcus* strain DN22 (Fournier et al. 2002) and *Rhodococcus* strains 11Y (Seth-Smith et al. 2002) and YH1 (Nejidat et al. 2008). RDX denitration has been coupled with spontaneous ring cleavage, with NDAB as a ring cleavage product (Bernstein and Ronen 2012). In addition, MEDINA has been observed as a ring cleavage product (Halasz et al. 2010). RDX denitration has been reported to be catalyzed by a cytochrome P450 enzyme (Coleman et al. 2002) and this has been demonstrated *in vivo* for the *Rhodococcus* strains DN22 (Bhushan et al. 2003; Coleman et al. 2002; Fournier et al. 2002), YH1 (Nejidat et al. 2008) and 11Y (Seth-Smith et al. 2008). The enzyme is encoded by the *xplA* gene (Seth-Smith et al. 2002) and has been found in RDX degrading *Rhodococcus* strains worldwide (Bernstein and Ronen 2012).

The enzymes XenA (in *Pseudomonas putida* II-B) and XenB (in *Pseudomonas fluorescens* I-C) can also transform RDX, with faster transformation being reported under anaerobic conditions . The authors noted that the primary degradation pathway was conversion to MEDINA then to formaldehyde, but a minor pathway also produced NDAB.

2.3. Classification of RDX Degrading Isolates

To date, known RDX degrading bacteria classify within the phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*. The majority of these isolates classify within the first three phyla with only one (HAW-EB21) within the *Fusobacteria* (Fuller et al. 2009; Zhao et al. 2004b).

The phylum *Firmicutes* contains RDX degrading bacteria within the classes *Clostridia* and *Bacilli*. This phylum contains the first anaerobic isolate reported to degrade RDX, *Clostridium bifermentans* (Regan and Crawford 1994). Other RDX degrading *Clostridia* isolates include *Clostridium* sp. EDB2 (Bhushan et al. 2004), *Clostridium acetobutylicum* (Zhang and Hughes 2003), *Clostridium bifermentans* HAW-1 and four other *Clostridia* isolates (Zhao et al. 2003). Additional isolates classifying as *Firmicutes* include *Acetobacterium malicum* sp. HAAP-1

(Adrian and Arnett 2004), *A. paludosum* (Sherburne et al. 2005), *Desulfitobacterium* (all in the class *Clostridia*) and the *Bacillus* strains HPB2 and HPB3 (Singh et al. 2009) (class *Bacilli*).

The phylum *Actinobacteria* (suborder *Corynebacterineae*) also contains numerous RDX degrading isolates including *Rhodococcus rhodochrous* 11Y (Seth-Smith et al. 2002), *Rhodococcus* sp. strain DN22 (Annamaria et al. 2010; Bhushan et al. 2003; Coleman et al. 1998; Coleman et al. 2002; Fournier et al. 2002), *Rhodococcus* strain YH1 (Nejdat et al. 2008), other *Rhodococcus* isolates (Bernstein et al. 2011; Seth-Smith et al. 2008), *Williamsia* sp. KTR4 (Thompson et al. 2005), *Gordonia* sp. KTR9 (Indest et al. 2010; Thompson et al. 2005) and *Gordonia* sp. YY1 (Ronen et al. 2008).

RDX degrading isolates in the phylum *Proteobacteria* are found within the *Alpha*-, *Gamma*- and *Deltaproteobacteria* classes. The majority of these classify as *Gammaproteobacteria* and include the genera *Serratia*, *Enterobacter*, *Citrobacter*, *Morganella*, *Klebsiella* and *Providencia* (order *Enterobacteriales*, family *Enterobacteriaceae*) (Pudge et al. 2003; Young et al. 1997; Zhao et al. 2002). Additional RDX degrading *Gammaproteobacteria* include the genera *Pseudomonas* (order *Pseudomonadales*, family *Pseudomonadaceae*) (Cho et al. 2008; Singh et al. 2009), *Shewanella* (order *Alteromonadales*, family *Shewanellaceae*) (Zhao et al. 2005; Zhao et al. 2006; Zhao et al. 2004b) and *Stenotrophomonas* (order *Xanthomonadales*, family *Xanthomonadaceae*) (Binks et al. 1995). RDX degrading isolates in the *Deltaproteobacteria* include the genera *Desulfovibrio* (order *Desulfovibrionales*, family *Desulfovibrionaceae*) (Arnett and Adrian 2009; Boopathy et al. 1998; Zhao et al. 2004b), *Anaeromyxobacter* (order *Myxococcales*, family *Cystobacteraceae*) (Kwon and Finneran 2008b) and *Geobacter* (order *Desulfuromonadales*, family *Geobacteraceae*) (Kwon and Finneran 2008a). Only one RDX degrading *Alphaproteobacteria* has been identified, this organism falls in genus *Methylobacterium* (order *Rhizobiales*, family *Methylobacteriaceae*) (Van Aken et al. 2004).

2.4. Source of RDX Degrading Isolates

RDX degrading bacteria have been isolated from a variety of sources. Many of these sources (e.g. soil, sediment or effluent) were previously exposed to explosives. However, other sources (e.g. compost soil, horse manure or poplar tissue) were not previously exposed to these contaminants. Collectively, this suggests RDX degraders are not limited to one ecosystem type.

Further, sources have included various geographical areas, including Canada, USA, Russia, United Kingdom, Australia, Israel and India. The following provides more detail on the source of the RDX degrading isolates discussed above.

The RDX degrading microorganism, HAW-EB21, (phylum *Fusobacteria*) was isolated using marine sediment from a former ammunition dumping site (Halifax Harbor) (Zhao et al. 2004a; Zhao et al. 2004b). RDX transforming isolates within the phylum *Firmicutes* have been isolated from a variety of sources and geographical areas. For example, *Clostridia bifermentans* was isolated from an anaerobic digester fed munitions compounds as the sole source of carbon and energy (Funk et al. 1993; Regan and Crawford 1994). *Clostridium* sp. strain EDB2 was isolated from marine sediment (Bhushan et al. 2004). Other *Clostridia* were obtained from an enrichment culture derived from a continuous anaerobic sludge blanket digester in Cornwall, Canada (Zhao et al. 2003). In contrast, the RDX degrading *Clostridium acetobutylicum* (ATCC 824) is a ubiquitous bacteria in soils and sediments (Zhang and Hughes 2003).

Other RDX degrading bacteria within *Firmicutes* include *Acetobacterium malicum* sp. HAAP-1 which was derived from a methanogenic mixed culture originating from the Holston Army Ammunition wastewater treatment plant (Kingsport, TN) (Adrian and Arnett 2004; Adrian et al. 2003; Hwang et al. 2000). *A. paludosum* was isolated from sediment of a marsh north of Moscow, Russia (Kotsyurbenko et al. 1995; Sherburne et al. 2005) and the *Bacillus* strains HPB2 and HPB3 were isolated from industrial effluent from a factory in India (Singh et al. 2009). In contrast, *Desulfitobacterium chlororespirans* strain Co23, was isolated from a compost soil (Kwon and Finneran 2008b; Sanford et al. 1996).

RDX degrading species within the phylum *Actinobacteria* have primarily been isolated from contaminated sources. For example, nineteen strains of *Rhodococcus* spp. were isolated from material collected from an explosive contaminated Ministry of Defense site in England (Seth-Smith et al. 2008). *Rhodococcus* sp. strain DN22 was isolated from explosives-contaminated soil in Australia (Coleman et al. 1998). Two other *Rhodococcus* strains (T7 and T9N) were isolated from sediments collected from Israel's coastal aquifer, which was contaminated with RDX (Bernstein et al. 2011). *Williamsia* sp. KTR4 and *Gordonia* sp. KTR9 were isolated from surface soil from the Naval Air Warfare Center Weapons Division in China Lake, Ca (prior exposure of this soil to explosives was possible) (Thompson et al. 2005). Further, the RDX degrading

bacteria *Gordonia* sp. YY1 was isolated from soil collected at a contaminated site north of Tel Aviv (Ronen et al. 2008).

RDX degrading bacteria within the phylum *Proteobacteria* have been isolated from both pristine and contaminated sources. RDX degrading *Gammaproteobacteria* from the genera *Morganella*, *Citrobacter* and *Providencia* were isolated from nitramine explosive contaminated soil. *Klebsiella pneumonia* strain SCZ-1 was isolated from a methanogenic industrial sludge (Zhao et al. 2002), *Pseudomonas putida* HK-6 was obtained from RDX contaminated soils (Chang et al. 2004; Cho et al. 2008) and *Pseudomonas* HPB1 was isolated from industrial effluent from a factory in India (Singh et al. 2009). RDX degrading bacteria classifying within the genus *Shewanella* were isolated using marine sediment from a former ammunition dumping site (Halifax Harbor) (Zhao et al. 2005; Zhao et al. 2006; Zhao et al. 2004b). *Stenotrophomonas maltophilia* PB1 was isolated from soil and water samples collected from a site that had been heavily contaminated with explosives (Binks et al. 1995). In contrast, *Serratia marcescens*, was isolated from horse manure (Young et al. 1997).

Within the *Deltaproteobacteria*, RDX degrading *Desulfovibrio* spp. have been isolated from creek sediment that was contaminated with explosives (Boopathy et al. 1998; Boopathy and Manning 1996) and from marine sediment from a former ammunition dumping site (Zhao et al. 2004b). Another *Deltaproteobacteria*, *Anaeromyxobacter dehalogenans* was isolated from anaerobic microcosms containing soil or sediment (Kwon and Finneran 2008b; Sanford et al. 2002). Interestingly, the only reported RDX degrading *Alphaproteobacteria*, *Methylobacterium* sp. strain BJ001, was isolated from hybrid poplar tissues (Van Aken et al. 2004).

Although much is known about these RDX degrading isolates, it is still uncertain if these organisms can perform RDX degradation at contaminated sites. The following section reviews RDX degrading microbial communities (primarily soil, aquifer and groundwater) to better understand the microorganisms potentially responsible for RDX degradation at contaminated sites.

2.5. RDX Degrading Mixed Communities

2.5.1. RDX Degrading Mixed Communities in Soil

Several studies have investigated the microbial communities present in RDX degrading samples derived from soil. The following reviews the microorganisms present in three studies involving

RDX degradation in 1) saturated and unsaturated soil (Ringelberg et al. 2008), 2) soil from different depths incubated aerobically and anaerobically (Ronen et al. 2008) and 3) soil with co-exposure to 2,4,6-trinitrotoluene (TNT) (Moshe et al. 2009). The classification of these organisms is also shown (Table 1).

2.5.1.1. Communities in Saturated vs. Unsaturated Soils

Microbial communities were compared in saturated vs. unsaturated soils (Ringelberg et al. 2008) using soil from Ft. Greely, AK, where past exposure to RDX was likely (Ringelberg et al. 2003). The RDX half-life was 4 days for saturated samples and 29 days for unsaturated microcosms. Terminal restriction fragment length polymorphism (TRFLP) was used to investigate microbial diversity.

The researchers reported a decreased number of bacterial species in the saturated soil (32 vs. 13 species) (Ringelberg et al. 2008). The saturated soil contained *Proteobacteria* (55%), *Firmicutes* (36%), *Actinobacteria* (8%) and *Bacteroidetes* (1%), with the *Proteobacteria* consisting of *Beta*- (44%), *Alpha*- (36%) and *Deltaproteobacteria* (20%). The unsaturated soil contained *Proteobacteria* (66%), *Firmicutes* (19%), *Chlorobi* (7%), *Actinobacteria* (6%) and *Bacteroidetes* (2%), with the *Proteobacteria* consisting of *Beta*- (63%) and *Gammaproteobacteria* (37%) only. Overall, the saturated soil illustrated a shift towards the phylum *Firmicutes*, and the classes *Alpha*- and *Deltaproteobacteria* and a shift away from *Gammaproteobacteria*.

The authors reported the saturated soil contained TRFLP fragments that corresponded to *Clostridia* (phylum *Firmicutes*), *Rhodococcus* (phylum *Actinobacteria*), and *Beijerinckia* (class *Alphaproteobacteria*) and these were not present in the unsaturated soil (Ringelberg et al. 2008). Other organisms including *Alpha*- and *Deltaproteobacteria* (genera *Myxococcus*, *Methylosinus*, *Rhodobacter* and *Sphingomonas*) were also unique to the saturated soil. As discussed above, *Clostridia* and *Rhodococcus* genera contain many isolates able to degrade RDX (Annamaria et al. 2010; Bernstein et al. 2011; Bhushan et al. 2004; Bhushan et al. 2003; Coleman et al. 1998; Coleman et al. 2002; Fournier et al. 2002; Nejdat et al. 2008; Regan and Crawford 1994; Seth-Smith et al. 2008; Zhang and Hughes 2003; Zhao et al. 2003). The authors also indicated that the genus *Beijerinckia* has been linked to RDX degradation. The two most abundant organisms in the saturated soil were *Sporosarcina urea* and *Halobacillus halophilus* (both in the phylum

Firmicutes, order *Bacillales*). Isolates have previously been linked to RDX degradation from the order *Bacillales*. In contrast, in the unsaturated soil *Burkholderia* (class *Betaproteobacteria*) was one of the most abundant species. These organisms have not previously been linked to RDX degradation.

2.5.1.2. RDX Degrading Aerobic and Anaerobic Soil Communities at Different Depths

Another study examined microbial populations in RDX degrading enrichments constructed with samples from different depths within the unsaturated zone (0 m, 1 m, 22 m and 45 m) (Ronen et al. 2008). The samples were obtained from a large military industrial complex north of Tel Aviv. The researchers exposed the samples to both aerobic and anaerobic conditions. Anaerobic RDX biodegradation occurred throughout the entire depth tested, with slower degradation in the deeper samples (both 0 m and 1 m samples were degraded by day 14, while 22 m and 45 m were only ~50% degraded by this time). In contrast, when the same samples were maintained under aerobic conditions, only the surface soil sample exhibited RDX degradation (complete degradation by day 7).

Using denaturing gradient gel electrophoresis (DGGE), the researchers found the microbial populations to be diverse and unique for each depth. The molecular analysis did not detect any known RDX degrading isolates within the families *Enterobacteriaceae* or *Clostridiaceae* or the suborder *Corynebacterineae*. The authors conclude that DGGE bands sequenced as *Pseudomonadales* (*Gammaproteobacteria*) and *Burkholderiales* (*Betaproteobacteria*) may have been important for RDX degradation. Of particular interest was a band that sequenced as a *Sphingomonas* sp. (*Alphaproteobacteria*), as this was found in all samples. These results are informative because RDX degrading isolates have not been previously reported from the orders *Sphingomonadales* or *Burkholderiales*. All organisms with a close identity to known species classified within the phyla *Proteobacteria* (*Alpha*-, *Beta*- or *Gamma*-), *Bacteroidetes*, or *Fibrobacteres*. Although no members of the *Gordoniaceae* family were detected in the molecular analysis, the authors isolated an aerobic RDX degrading microorganism from soil surface sample enrichment cultures and found it to be closely related to the genus *Gordonia*.

2.5.1.3. Soil RDX Degrading Communities Exposed to TNT

The effect of TNT on the soil microbial community has also been examined (Moshe et al. 2009). The contaminated soil was obtained from below the surface of an infiltration pond that had been used for untreated wastewater from explosives-manufacturing plants. When RDX was present alone, complete degradation was noted after 18 days, however, when it was amended with a mixture of other explosives (TNT and HMX) complete degradation required 28 days. Using DGGE, the authors reported that TNT exposure resulted in a reduction in microbial abundance and diversity. One DGGE band survived all treatments and exhibited 92% similarity to *Clostridium nitrophenolicum*. Another DGGE band was present in all but one treatment and exhibited 97% similarity to *Clostridium kluyveri* DSM 555. These results suggest that organisms within the class *Clostridia* are important for RDX degradation in these samples.

2.5.2. RDX Degrading Microbial Communities in Groundwater and Aquifer Samples

Several groups have used groundwater (and in some cases, aquifer material) from contaminated sites to investigate the microbial communities associated with RDX degradation. The majority of these studies were performed with samples from Picatinny Arsenal (NJ) (Kwon and Finneran 2010; Kwon et al. 2011; Roh et al. 2009). One group studied samples from two sites subject to different bioremediation approaches. Two projects used stable isotope probing (SIP) to more precisely define the RDX degrading microorganisms (Cho et al. 2013; Roh et al. 2009). Another group investigated the effect of substrate addition and electron shuttling chemicals on RDX degrading communities (Kwon and Finneran 2010; Kwon et al. 2011). The most recent report investigated temporal changes in groundwater communities following biostimulation at a contaminated site (Livermore et al. 2013). The microbial communities determined by these researchers are discussed in turn below. The classification of these organisms is also shown (Table 2).

2.5.2.1. Contaminated Site Groundwater and RDX Enrichments Communities

The microbial community was examined in groundwater from two sites where RDX bioremediation was being tested (Fuller et al. 2010). One site involved cheese whey addition to promote the biostimulation of RDX (Picatinny Arsenal, NJ). A mulch biowall was being used at the other site to promote RDX biodegradation (Pueblo Chemical Depot, CO). Samples were

collected upgradient and downgradient from the biostimulation areas. Aquifer sediments from Picatinny Arsenal were used in a laboratory column study which involved contaminated groundwater from the site. Column effluent samples were used to construct enrichment cultures. DNA was extracted from column effluent, enrichment cultures and groundwater (both native and biostimulated). The microbial community analysis of the extracted DNA included both DGGE and 16S rRNA gene sequencing.

From all sequences, the majority classified as *Proteobacteria* (52%) and these were primarily *Alpha*- and *Gammaproteobacteria*. The other sequences classified as *Firmicutes* (25%), *Bacteroidetes*, *Actinobacteria* (both totaled to 6%) and a portion could not be classified (17%). *Gammaproteobacteria* dominated in the aqueous enrichment cultures. The native groundwater was dominated by *Alpha*- and *Gammaproteobacteria*. The biostimulated groundwater was dominated by *Gammaproteobacteria*, *Bacteroidetes* and *Firmicutes*.

The researchers found a wide variety of bacteria, however, did not find any exact matches to known RDX degraders. The closest matches to known RDX degraders were those that classified as *Pseudomonas* and *Clostridium* and these were also the most common sequences. Multiple sequences obtained from the enrichments and column effluent classified as *Azospira* (*Betaproteobacteria*). *Pseudomonas* sequences were found in the enrichment cultures derived either from the Picatinny Arsenal groundwater or from the column effluent (Picatinny Arsenal groundwater as influent). No such sequences were found from the groundwater itself (native or biostimulated) or directly from the column effluent. However, several sequences identified as *Pseudomonas* were found in groundwater from the other site. *Clostridium* sequences were identified approximately equally from all sample types (except one set of enrichments). The authors stated this genus is likely associated with the degradation of RDX on site. The authors also suggest that a wider variety of bacteria may be responsible for RDX degradation in groundwater over and above what is known from RDX degrading isolates.

2.5.2.2. Stable Isotope Probing on Contaminated Site Groundwater and Microcosms

One group used a more direct method to identify organisms responsible for RDX degradation in contaminated groundwater. The research involved stable isotope probing (SIP) and the identification of organisms assimilating ^{15}N from ^{15}N labeled RDX (Roh et al. 2009). The study was performed on samples (soil and groundwater) from the Picatinny Arsenal site.

Microcosms, amended with labeled RDX, cheese whey, and column (constructed from site samples) effluent were initially aerobic and were allowed to become anoxic. Fifteen 16S rRNA sequences were obtained from the ^{15}N DNA fraction (therefore organisms that had incorporated the label from RDX). These organisms included *Gammaproteobacteria* (six sequences), *Alphaproteobacteria* (seven sequences) and *Actinobacteria* (two sequences). Three were similar to *Azospirillum* sp. (*Alphaproteobacteria*) and two were similar to the genus *Pseudomonas*. There were five sequences that were highly similar to two known RDX degraders (*Pseudomonas fluorescens* I-C and *Enterobacter cloacae*). The other ten were not similar to known RDX degraders.

The 16S rRNA genes present in the RDX contaminated groundwater were also examined. The 25 unique sequences obtained classified with the phyla *Proteobacteria* (primarily *Alphaproteobacteria*), *Firmicutes* and *Actinobacteria*. Only one sequence from the groundwater was identified as a *Clostridium* species. Most of the groundwater gene sequences were not previously linked to RDX degradation and were not similar to those found in the microcosm study.

Another SIP study, by the same group, used ^{13}C labeled RDX to investigate microbial diversity in RDX degrading microcosms containing groundwater, with and without cheese whey addition (Cho et al. 2013). Microcosms were created using groundwater from two wells (US army facility in northern New Jersey) and were amended with labeled RDX. Faster RDX degradation was observed in microcosms amended with cheese whey (30 days compared to 35-300 days). The organisms assimilating the label were determined by 16S rRNA gene sequencing of the label enriched DNA.

Overall, the diversity of organisms was higher without cheese whey addition. In these samples, for one groundwater microcosm, ten different 16S rRNA gene sequences were identified. These classified within the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Spirochaetes*. For the other groundwater microcosm, six sequences were obtained, classifying within the *Bacteroidia*, *Clostridia*, *Betaproteobacteria* and *Spirochaetes*. From the 16 sequences obtained, the majority (11 sequences) classified within the *Bacteroidia*, *Betaproteobacteria* and the *Spirochaetes* and this is the first report of RDX degradation in these taxonomic groups. Two sequences showed similarity to a *Rhodoferrax* sp. and these were found to be important in another RDX study involving contaminated site material

(below) (Kwon et al. 2011). Other sequences were similar to organisms previously associated with RDX degradation. For example, one sequence fell within the class *Clostridia* and two sequences classified as *Desulfovibrio* species. Two sequences classified within the *Rhizobiales* (*Alphaproteobacteria*).

The microbial community involved in RDX consumption was different when cheese whey was added. In microcosms derived from one groundwater sample, the 14 labeled clones identified within the *Gammaproteobacteria* and *Bacilli*. The seven *Gammaproteobacteria* were all *Pseudomonas* sp. and clustered with two known RDX degraders. From the microcosm amended with the other groundwater well sample and cheese whey, 10 different clones were obtained. These classified within three phyla, *Betaproteobacteria*, *Actinobacteria* and *Firmicutes*.

2.5.2.3. RDX Degrading Communities in Aquifer and Groundwater Samples

The effect of substrate addition and electron shuttling chemicals on the microbial community of RDX degrading samples was recently examined (Kwon and Finneran 2010; Kwon et al. 2011). The aquifer material (sediment and groundwater) was obtained from Picatinny Arsenal (NJ). In one study, either lactate or acetate was added to microcosms with sediment, groundwater and RDX (Kwon et al. 2011). Initially, acetate or lactate was added at stoichiometric concentrations for the reduction of RDX. As both were depleted during the experiment, additional acetate or lactate was added at day 34. RDX degradation was faster in the acetate or lactate amended samples compared to the no donor controls (degradation by day 60). The authors found that acetate stimulated Fe (III) reduction and lactate stimulated both Fe (III) reduction and sulfate reduction.

Clone libraries of 16S rRNA sequences were constructed before and after day 34 for both amendment types. The authors reported that before day 34, sequences belonging to *Alpha*-, *Beta*-, and *Gammaproteobacteria* were dominant and after this time *Betaproteobacteria* were dominant. They also reported that the microbial community was strongly affected by the electron donor added. Specifically, they found that acetate enriched for *Rhodoferrax* spp. (*Betaproteobacteria*) and *Anaeromyxobacter* spp. (*Deltaproteobacteria*), while lactate enriched for *Rhodoferrax* spp. and *Clostridia* spp. (phylum *Firmicutes*). Previous work reported *Rhodoferrax* are Fe (III) reducing bacteria that can use both acetate and lactate as electron donors

(Finneran et al. 2003). *Anaeromyxobacter* spp. have previously been reported to reduce RDX (Kwon and Finneran 2008b), can use Fe (III) as an electron acceptor and acetate as an electron donor (Sanford et al. 2002).

In another study by the same group, material from the same site was used to investigate the microbial communities in microcosms amended with acetate and electron shuttling compounds (humic acid, HS and anthraquinone-2-6-disulfonate, AQDS) (Kwon and Finneran 2010). The authors noted that RDX (^{14}C labelled) mineralization (determined via $^{14}\text{CO}_2$ analysis) was higher in the AQDS and HS amended samples. The researchers found that unique Fe (III) reducing microbial communities (*Beta*- and *Gammaproteobacteria*) dominated in shuttle amended samples. The authors reported that the extracellular electron shuttling increased the rate and extent of RDX reduction without significant accumulation of undesirable intermediates. *Alphaproteobacteria* (genus *Ochrobactrum*) were dominant at day 0 and remained a significant part of the community in all incubations. AQDS amended incubation samples included *Pseudomonas* and *Geobacteraceae*. In the incubations with HS a significant percentage of (52%) the sequences were *Betaproteobacteria* (family *Oxalobacteraceae*). In acetate only incubations, 30% were related to *Betaproteobacteria* (families *Oxalobacteraceae* and *Comamonadaceae*) and 8% related to *Gammaproteobacteria* (genus *Pseudomonas*).

2.5.2.4. Biostimulated Groundwater Communities

A recent study used pyrosequencing to investigate temporal changes in the microbial community structure in groundwater at an RDX contaminated site (near the Iowa Army Ammunition Plant, Middletown, IA) where biostimulation was being performed (Livermore et al. 2013). The remediation approach involved the addition of acetate. After the first acetate addition, the groundwater community in the injection well was dominated by *Betaproteobacteria* (>90% of the community). After two and four months, respectively, the majority classified as *Rhodocyclales* (54% of *Betaproteobacteria*) and *Burkholderiales* (87% of *Betaproteobacteria*). The authors reported many of these sequences are closely related to known Fe (III) reducing bacteria. Following another acetate addition and during the period when most significant RDX degradation occurred, the microbial community was dominated by *Deltaproteobacteria* (54% and 63% in two samples) and *Bacteroidetes* (25% in two samples). Also the *Betaproteobacteria* was reduced to 3% in both samples. The significant percentage of the *Deltaproteobacteria* in two

of three samples (taken after the second acetate addition) classified as *Geobacteraceae*. The majority of *Bacteroidetes* classified as *Flavobacteriales* and others were placed in *Chitinophagaceae* and *Saprospiraceae*. When RDX reduction had stopped, the *Betaproteobacteria* became dominant again.

The authors also analyzed other wells using TRFLP (34 samples from 14 wells). The authors regressed the TRF abundance and RDX metabolite detection in these wells and found that the proportion of Fe (III) reducers that were *Deltaproteobacteria* (*Geobacter* spp.) was the most significant predictor variable. The authors suggest a link between *Geobacter* spp. and RDX nitroso-metabolite accumulation.

2.5.3. RDX Degrading Microbial Communities in Non-Contaminated Site Studies

A few studies have investigated microbial communities in RDX degrading samples that were not derived from contaminated sites. One project examined communities in samples derived from RDX wastewater treatment plant samples (Arnett et al. 2009). Another study used SIP to investigate RDX degrading communities in soil not previously exposed to RDX (Jayamani et al. 2010). Finally, two studies focused on RDX degrading communities in ovine rumen fluid (Eaton et al. 2011; Perumbakkam and Craig 2012). The key findings of these projects are summarized below. The classification of these organisms is also shown (Table 3).

2.5.3.1. RDX Wastewater Treatment Plant Derived Microbial Community

Microbial communities were compared in enrichment cultures (derived from RDX wastewater treatment plant samples, Holston Army Ammunition Plant in Kingsport, TN) with and without sulfate addition (Adrian et al. 2003; Arnett et al. 2009). RDX was completely degraded in the sulfate amended samples (media and enrichment culture) by day 3, however, in the unamended samples, 65% of RDX remained during the same time period.

Sequencing of 16S rRNA genes from the original enrichment indicated the majority of the identified clones were *Geobacter* (78%), and others included *Clostridia* (13%, unclassified) and *Clostridiaceae* (4%, unclassified). The remaining clones were unclassified bacteria, a *Cryptanaerobacter* sp. and an additional unclassified *Clostridiaceae*. Further enrichments on this culture resulted in the identification of a *Desulfovibrio* sp., a *Sulfuricurvum* sp. and an *Acetobacterium* sp.

In the RDX degrading sulfate unamended samples, TRFLP fragments identified as *Geobacter* comprised ~85% of the initial TRFLP distribution. At day 3 and day 7, TRFLP analysis indicated this species accounted for 56% and 70% of the community. The second most abundant organism at the start of the study was an *Acetobacterium* sp. (started at 10% and increased to 24% by day 3, then slowly decreased). Other species (*Sulfuricurvum* and *Desulfovibrio*) were detected in the TRFLP profile, but their abundance was low throughout the RDX degradation period.

The authors previously isolated the RDX degrading organism *Acetobacterium malicum* strain HAAP-1 from this culture (Adrian and Arnett 2004). However, in the enriched culture they concluded it had limited influence because it was present at low levels (2% of TRFLP total abundance) and did not illustrate any growth in numbers until RDX was depleted. Instead the authors suggest the other *Acetobacterium* sp. was involved in RDX degradation under autotrophic conditions. The researchers also hypothesized that the *Geobacter* sp. was involved in direct electron shuttling which could have contributed to RDX degradation, as this has been previously documented (Kwon and Finneran 2006).

The addition of sulfate changed the community profile of the RDX degrading culture. The *Geobacter* and *Acetobacterium* clones (dominant in the sulfate amended samples) decreased to <3% by day 3. An unclassified *Clostridiaceae* clone became the dominant organism by day 3. Also, a *Sulfuricurvum* sp. (initially present at ~3%) was the most dominant at day 7 and a *Desulfovibrio* sp. (undetected in the original TRFLP profile) became dominant by day 14. RDX-degrading *Desulfovibrio* species have previously been identified (Arnett and Adrian 2009; Zhao et al. 2004b; Zhao et al. 2003). Information on the metabolic abilities of *Sulfuricurvum* sp. are lacking and so it is unknown if this organism is involved in RDX degradation.

2.5.3.2. RDX Degrading Communities in Pristine Soil

SIP with ¹³C and ¹⁵N labelled RDX was used to directly identify the organisms responsible for RDX assimilation in a pristine soil (no previous exposure to RDX) (Jayamani et al. 2010). When this soil was aerated daily no RDX degradation occurred. However, when oxygen depletion occurred, RDX was degraded within 14 days. For the SIP study, soil microcosms were amended with labelled RDX and following RDX degradation, DNA was extracted, ultracentrifuged, then

TRFLP was performed on the heavy fractions (containing the labelled DNA). In addition, a 16S rRNA gene clone library was generated following RDX degradation.

The TRFLP data from the ultracentrifugation fractions indicated one TRFLP fragment was responsible for label uptake. Further analysis revealed this fragment was belonged to organisms in the order *Sphingobacteriales* (phylum *Bacteroidetes*) or the phylum *Acidobacteria*. To date, these organisms have not previously been linked to RDX degradation. The 16S rRNA gene clone library was also unique compared to the RDX degrading mixed communities described above. The library primarily consisted of *Acidobacteria* (36%), *Proteobacteria* (28%) and *Bacteroidetes* (15%).

2.5.3.3. RDX Degrading Rumen Microbial Communities

Two studies have investigated microbial communities in RDX degrading samples originating from ovine rumen (Eaton et al. 2011; Perumbakkam and Craig 2012). The first involved an examination of the microbial community in whole ovine rumen fluid in low nitrogen basal media and methanogenic media (Eaton et al. 2011). In these samples, RDX degradation occurred rapidly (< 7 days). In the low nitrogen basal medium, sequences classified within the phyla *Eurarchaeota*, *Actinobacteria* (genera *Olsenella* and *Actinomyces*) and *Firmicutes* (genus *Lactobacillus*). In the methanogenic media, one sequence was also similar to the *Olsenella* genus and the other four classified within the phylum *Firmicutes* (*Clostridium*, *Sporanaerobacter* and *Streptococcus* genera). Overall, *Actinobacteria* and *Firmicutes* were enriched in the RDX degrading low nitrogen basal medium and methanogenic medium respectively. The authors also enriched the RDX degrading methanogenic consortium and found all clones to be identical to the *Sporanaerobacter* or *Clostridium* organisms identified in their earlier studies. This is the first report of linking organisms in the genus *Sporanaerobacter* to RDX degradation.

The second rumen RDX study assessed microbial diversity over time in ovine rumen derived samples (Perumbakkam and Craig 2012). This study also demonstrated rapid removal of RDX (8 hours). The experiment involved DNA extraction and analysis at various times (0, 4 and 8 hours) from whole rumen fluid samples amended with RDX. The authors reported complete RDX degradation within 8 hrs and at this time the majority of sequences were associated with the phyla *Bacteroidetes* (46-54%) and *Firmicutes* (38-39%). Organisms classifying within the phylum TM7 were also present after 8 hours, however at a lower level (3.8-8.2%). The

rarefaction curves showed no trend towards reaching a plateau indicating not enough clones were sequenced to characterize all of the diversity within their samples. When the authors compared the clone libraries from the samples amended with RDX over time, they found a significant decrease for the clones associated with TM7 and *Clostridia*. They also found a significant increase in clones associated with *Prevotella* sp. (phylum *Bacteroidetes*). *Prevotella* strains are considered to be the most dominant bacteria in the rumen (Stevenson and Weimer 2007). The results indicate that members of the genus *Prevotella* were important for RDX degradation in these experiments.

2.6. Prediction of Key *In Situ* RDX Degradors

The reported organisms in the RDX degrading mixed cultures or environmental samples are summarized (Tables 1, 2 and 3). Along with pure culture data, tables 1 and 2 (nine studies all using contaminated site samples) were used to create hypotheses on which organisms are likely to be important for *in situ* RDX degradation, as discussed below.

Firmicutes were reported in the majority (six from nine) of the studies that used contaminated site samples (Cho et al. 2013; Fuller et al. 2010; Kwon et al. 2011; Moshe et al. 2009; Ringelberg et al. 2008; Roh et al. 2009). From these, organisms in the class *Clostridia* were noted to be dominant in many of these studies (Fuller et al. 2010; Kwon et al. 2011; Moshe et al. 2009; Roh et al. 2009). Only in two cases were these sequences classified further, to the family (*Clostridiaceae 1*) (Moshe et al. 2009) or to the genus level (*Anaerobacter*) (Cho et al. 2013). Organisms in the class *Bacilli* were also noted to be dominant in several studies (Cho et al. 2013; Ringelberg et al. 2008). These organisms were further classified (to the genera *Sporosarcina* and *Halobacillus*) (Ringelberg et al. 2008), *Sporolactobacillus* (Fuller et al. 2010) or *Trichococcus* (Cho et al. 2013). The occurrence of *Clostridia* and *Bacilli* in contaminated site samples and the previous reports of RDX degrading *Firmicutes* isolates suggest these are key organisms for *in situ* RDX degradation.

Actinobacteria were reported in many (five from nine) of the studies that used contaminated site samples (Cho et al. 2013; Fuller et al. 2010; Ringelberg et al. 2008; Roh et al. 2009; Ronen et al. 2008). These organisms classified to the genera *Rhodococcus* (Cho et al. 2013; Ringelberg et al. 2008), *Gordonia* (Ronen et al. 2008), *Cellulomonas* (Cho et al. 2013). In some cases, further classification was not provided. As RDX degrading isolates also classify within the genera

Rhodococcus and *Gordonia* it is likely these genera are important for degrading RDX at contaminated sites.

The majority of RDX degrading isolates within the phylum *Proteobacteria* classify within the *Gammaproteobacteria* class (section 2). Organisms in this class were also reported in the majority (six from nine) of the contaminated site studies (Cho et al. 2013; Fuller et al. 2010; Kwon and Finneran 2010; Ringelberg et al. 2008; Roh et al. 2009; Ronen et al. 2008). When further classification was provided, these organisms fell within the orders *Pseudomonadales*, (Cho et al. 2013; Fuller et al. 2010; Kwon and Finneran 2010; Ringelberg et al. 2008; Roh et al. 2009; Ronen et al. 2008), *Legionellales* (Ronen et al. 2008), *Enterbacterales* (Roh et al. 2009). Significantly, in several cases, organisms classifying as *Pseudomonadales* were dominant in RDX degrading contaminated site derived samples (Cho et al. 2013; Fuller et al. 2010; Ronen et al. 2008). Again, as many RDX degrading isolates fall within the orders *Pseudomonadales* and *Enterbacterales*, it would be reasonable to hypothesize that these organisms will be important for *in situ* RDX degradation.

Deltaproteobacteria were also reported in many (five from nine) of the studies shown in tables 1 and 2 (Cho et al. 2013; Kwon and Finneran 2010; Kwon et al. 2011; Livermore et al. 2013; Ringelberg et al. 2008). When the actual sequences were provided, the organisms classified within the orders *Myxococcales* (Kwon et al. 2011; Ringelberg et al. 2008), *Desulfovibrionales* (Cho et al. 2013), *Desulfuromonadales* (Kwon and Finneran 2010; Livermore et al. 2013). All three orders contain known RDX degrading isolates, therefore, all are likely relevant for *in situ* RDX degradation.

Alphaproteobacteria were reported in six of the nine studies (Cho et al. 2013; Fuller et al. 2010; Kwon and Finneran 2010; Ringelberg et al. 2008; Roh et al. 2009; Ronen et al. 2008). When provided, the sequences classified within the orders *Rhizobiales* (Cho et al. 2013; Kwon and Finneran 2010; Ringelberg et al. 2008), *Rhodobacterales* (Ringelberg et al. 2008), *Sphingomonadales* (Ringelberg et al. 2008; Ronen et al. 2008) and *Rhodospirillales* (Roh et al. 2009). Only in two cases, were these organisms (*Sphingomonadales* and *Rhizobiales*) reported to be dominant (Kwon and Finneran 2010; Ronen et al. 2008). The only known RDX degrading *Alphaproteobacteria* is within the class *Rhizobiales*. Given the lack of dominance of this class across all studies and the limited number of RDX degrading isolates, it is reasonable to hypothesize these organisms may not be common RDX degraders *in situ*.

In several studies, dominant organisms classified within phyla with no known RDX degrading isolates. The most common case was for the phylum *Betaproteobacteria*. Five studies reported a dominance of organisms in the order *Burkholderiales* (Cho et al. 2013; Kwon and Finneran 2010; Kwon et al. 2011; Ringelberg et al. 2008; Ronen et al. 2008). Other studies reported the presence (not dominance) of *Betaproteobacteria* within the order *Rhodocyclales* (Cho et al. 2013; Fuller et al. 2010; Livermore et al. 2013) or *Burkholderiales* (Livermore et al. 2013). The common dominance of *Burkholderiales* across studies could potentially indicate their importance to *in situ* RDX degradation. Organisms within the phylum *Bacteroidetes* (also contains no known RDX degrading isolates) were reported in some studies (Cho et al. 2013; Fuller et al. 2010; Livermore et al. 2013; Ringelberg et al. 2008; Ronen et al. 2008), however, only in one case were these organisms reported to be dominant (order *Flavobacteria*) (Livermore et al. 2013). In one study, organisms within the phylum *Spirochaetes* reported to be dominant (Cho et al. 2013). Organisms in the phyla *Chlorobia* and *Fibrobactere* were reported to be present (but not dominant) (Ringelberg et al. 2008; Ronen et al. 2008). All four phyla have no known RDX degrading isolates. Given the low frequency across studies and the lack of dominance in any study it is unlikely organisms in these phyla are key RDX degraders.

In summary, a wide variety of organisms have been reported to be present and are likely responsible for RDX degradation at contaminated sites. From the phyla with known RDX degrading isolates, *Firmicutes* and *Proteobacteria* (particularly *Gammaproteobacteria*) were the most dominant organisms in many samples. Organisms in the phyla *Deltaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* were dominant in these studies less frequently. Notably, organisms within the class *Betaproteobacteria* were dominant in many samples and yet this class does not appear to contain any known RDX degraders. Therefore, this class could represent a group of novel RDX degraders, previously undiscovered based on pure culture work. Finally, organisms in the phyla *Bacteroidetes*, *Chlorobia*, *Fibrobactere* or *Spirochaetes* are likely not key degraders at contaminated sites.

Table 2.1. The classification of organisms in RDX degradation studies using primarily contaminated site soil or sediment. The organisms displayed in bold font were dominant in those samples.

Source	Treatment	Phylum	Class	Order	Family	Genus	Reference
Contaminated Surface Soil	Saturated (by phyla)	Proteobacteria (55%) Proteobacteria (55%) Proteobacteria (55%) Firmicutes (36%) <i>Actinobacteria (8%)</i> <i>Bacteroidetes (15%)</i>	Betaproteobacteria (44%) Alphaproteobacteria (36%) Deltaproteobacteria (20%)				(Ringelberg et al. 2008)
	Saturated (key sequences)	Firmicutes Firmicutes <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Firmicutes</i> <i>Actinobacteria</i>	Bacilli Bacilli <i>Deltaproteobacteria</i> <i>Alphaproteobacteria</i> <i>Alphaproteobacteria</i> <i>Alphaproteobacteria</i> <i>Clostridia</i> <i>Actinobacteria</i>	Bacillales Bacillales <i>Myxococcales</i> <i>Rhizobiales</i> <i>Rhizobiales</i> <i>Rhodobacterales</i> <i>Sphingomonadales</i> <i>Unclassified</i> <i>Actinomycetales</i>	Planococcaceae Bacillaceae 2 <i>Myxococcaceae</i> <i>Methylocystaceae</i> <i>Beijerinckiaceae</i> <i>Rhodobacteraceae</i> <i>Sphingomonadaceae</i> <i>Unclassified</i> <i>Nocardiaceae</i>	Sporosarcina urea Halobacillus halophilus <i>Myxococcus</i> <i>Methylosinus</i> <i>Beijerinckia</i> <i>Rhodobacter</i> <i>Sphingomonas</i> <i>Unclassified</i> <i>Rhodococcus</i>	
	Unsaturated (by phyla)	Proteobacteria (66%) Proteobacteria (66%) Firmicutes (19%) <i>Chlorobi (7%)</i> <i>Actinobacteria (6%)</i> <i>Bacteroidetes (2%)</i>	Betaproteobacteria (63%) Gammaproteobacteria (37%)				
	Unsaturated	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	
Contaminated Site Surface And Vadose Zone Soil		Proteobacteria Proteobacteria Proteobacteria <i>Proteobacteria</i> <i>Bacteroidetes</i> <i>Fibrobactere</i> <i>Proteobacteria</i>	Alphaproteobacteria Gammaproteobacteria Betaproteobacteria <i>Betaproteobacteria</i> <i>Unclassified</i> <i>Unclassified</i> <i>Gammaproteobacteria</i>	Sphingomonadales Pseudomonadales Burkholderiales <i>Burkholderiales</i> <i>Unclassified</i> <i>Unclassified</i> <i>Legionellales</i>	Sphingomonadaceae Unclassified Comamonadaceae <i>Alcaligenaceae</i> <i>Unclassified</i> <i>Unclassified</i> <i>Legionellaceae</i>	Sphingomonas Unclassified Unclassified <i>Unclassified</i> <i>Unclassified</i> <i>Unclassified</i> <i>Unclassified</i>	(Ronen et al. 2008)
	Isolate	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Gordonia	
Contaminated Soil		Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Unclassified Clostridium	(Moshe et al. 2009)
		Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Unclassified Clostridium	

Table 2.2. The classification of organisms in RDX degradation studies using primarily contaminated site groundwater. The organisms displayed in bold font were dominant in those samples.

Source	Treatment	Phylum	Class	Order	Family	Genus	Reference
Groundwater & Enrichment Cultures Before and After Biostimulation	(by phyla)	<i>Proteobacteria</i> (52%) <i>Proteobacteria</i> (52%) <i>Firmicutes</i> (25%) Unclassified (17%) <i>Bacteroidetes</i> & <i>Actinobacteria</i> (6%)	<i>Alphaproteobacteria</i> <i>Gammaproteobacteria</i>				(Fuller et al. 2010)
	(key sequences)	<i>Firmicutes</i> <i>Proteobacteria</i> <i>Firmicutes</i> <i>Proteobacteria</i>	<i>Clostridia</i> <i>Gammaproteobacteria</i> <i>Bacilli</i> <i>Betaproteobacteria</i>	<i>Unclassified</i> <i>Pseudomonadales</i> <i>Bacillales</i> <i>Rhodocyclales</i>	<i>Unclassified</i> <i>Pseudomonadaceae</i> <i>Sporolactobacillaceae</i> <i>Rhodocyclaceae</i>	<i>Unclassified</i> <i>Unclassified Pseudomonas</i> <i>Sporolactobacillus</i> <i>Azospira</i>	
Contaminated Site Aquifer & Groundwater	Microcosms Derived from Contaminated Site Material (¹⁵ N RDX SIP Study)	<i>Actinobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i>	<i>Gammaproteobacteria</i> <i>Alphaproteobacteria</i> <i>Gammaproteobacteria</i> <i>Gammaproteobacteria</i> <i>Alphaproteobacteria</i>	<i>Pseudomonadales</i> <i>Enterobacteriales</i> <i>Rhodospirillales</i>	<i>Pseudomonadaceae</i> <i>Enterobacteriaceae</i> <i>Rhodospirillaceae</i>	<i>Pseudomonas</i> <i>Enterobacter</i> <i>Azospirillum</i>	(Roh et al. 2009)
	Groundwater	<i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Firmicutes</i> <i>Actinobacteria</i>	<i>Alphaproteobacteria</i> <i>Betaproteobacteria</i> <i>Epsilonproteobacteria</i> <i>Gammaproteobacteria</i> <i>Clostridia</i>				
Groundwater from Contaminated Site	No Cheese Whey (¹³ C RDX SIP Study)	<i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Spirochaetes</i> <i>Bacteroidetes</i> <i>Firmicutes</i>	<i>Alphaproteobacteria</i> <i>Betaproteobacteria</i> <i>Betaproteobacteria</i> <i>Betaproteobacteria</i> <i>Betaproteobacteria</i> <i>Deltaproteobacteria</i> <i>Spirochaetes</i> <i>Bacteroidia</i> <i>Clostridia</i>	<i>Rhizobiales</i> <i>Burkholderiales</i> <i>Burkholderiales</i> <i>Rhodocyclales</i> <i>Rhodocyclales</i> <i>Hydrogenophilales</i> <i>Desulfovibrionales</i> <i>Spirochaetales</i> <i>Bacteroidales</i> <i>Clostridiales</i>	<i>Comamonadaceae</i> <i>Oxalobacteraceae</i> <i>Rhodocyclaceae</i> <i>Rhodocyclaceae</i> <i>Hydrogenophilaceae</i> <i>Desulfovibrionaceae</i> <i>Spirochaetaceae</i> <i>Rikenellaceae</i> <i>Clostridiaceae 1</i>	<i>Rhodoferax</i> <i>Undibacterium</i> <i>Sulfuritalea</i> <i>Ferribacterium</i> <i>Sulfuricella</i> <i>Desulfovibrio</i> <i>Treponema</i> <i>Rikenella</i> <i>Anaerobacter</i>	(Cho et al. 2013)
	With Cheese Whey (¹³ C RDX SIP Study)	<i>Firmicutes</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Actinobacteria</i> <i>Actinobacteria</i> <i>Actinobacteria</i>	<i>Bacilli</i> <i>Betaproteobacteria</i> <i>Gammaproteobacteria</i> <i>Actinobacteria</i> <i>Actinobacteria</i> <i>Actinobacteria</i>	<i>Lactobacillales</i> <i>Burkholderiales</i> <i>Pseudomonadales</i> <i>Actinomycetales</i> <i>Actinomycetales</i> <i>Actinomycetales</i>	<i>Carnobacteriaceae</i> <i>Oxalobacteraceae</i> <i>Pseudomonadaceae</i> <i>Propionibacteriaceae</i> <i>Cellulomonadaceae</i> <i>Nocardiaceae</i>	<i>Trichococcus</i> <i>Undibacterium</i> <i>Pseudomonas</i> <i>Unclassified</i> <i>Cellulomonas</i> <i>Rhodococcus</i>	
Contaminated Site Aquifer & Groundwater	Acetate Acetate Lactate Lactate	<i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Firmicutes</i>	<i>Betaproteobacteria</i> <i>Deltaproteobacteria</i> <i>Betaproteobacteria</i> <i>Clostridia</i>	<i>Burkholderiales</i> <i>Myxococcales</i> <i>Burkholderiales</i>	<i>Comamonadaceae</i> <i>Cystobacteraceae</i> <i>Comamonadaceae</i>	<i>Rhodoferax</i> <i>Anaeromyxobacter</i> <i>Rhodoferax</i>	(Kwon et al. 2011)
Contaminated Site Aquifer & Groundwater	Electron Shuttling Studies	<i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i> <i>Proteobacteria</i>	<i>Alphaproteobacteria</i> <i>Gammaproteobacteria</i> <i>Deltaproteobacteria</i> <i>Betaproteobacteria</i> <i>Betaproteobacteria</i>	<i>Rhizobiales</i> <i>Pseudomonadales</i> <i>Desulfuromonadales</i> <i>Burkholderiales</i> <i>Burkholderiales</i>	<i>Brucellaceae</i> <i>Pseudomonadaceae</i> <i>Geobacteraceae</i> <i>Oxalobacteraceae</i> <i>Comamonadaceae</i>	<i>Ochrobactrum</i> <i>Pseudomonas</i> <i>Geobacter</i>	(Kwon and Finneran 2010)
Groundwater From		<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfuromonadales</i>	<i>Geobacteraceae</i>	<i>Geobacter</i>	(Livermore et al. 2013)

Contaminated Site During Biostimulation		<i>Bacteroidetes</i> <i>Bacteroidetes</i> <i>Bacteroidetes</i> <i>Proteobacteria</i> <i>Proteobacteria</i>	<i>Flavobacteria</i> <i>Sphingobacteria</i> <i>Sphingobacteria</i> <i>Betaproteobacteria</i> <i>Betaproteobacteria</i>	<i>Flavobacteriales</i> <i>Sphingobacteriales</i> <i>Sphingobacteriales</i> <i>Rhodocyclales</i> <i>Burkholderiales</i>	<i>Chitinophagaceae</i> <i>Saprospiraceae</i>	
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Table 2.3. The classification of organisms in RDX degradation studies using material not derived from a contaminated site . The organisms displayed in bold font were dominant in those samples.

Source	Treatment	Phylum	Class	Order	Family	Genus	Reference
RDX WWTP	SO ₄ ²⁻ unamended	<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfuromonadales</i>	<i>Geobacteraceae</i>	<i>Geobacter</i>	(Arnett et al. 2009)
	SO ₄ ²⁻ unamended	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Eubacteriaceae</i>	<i>Acetobacterium</i>	
	SO ₄ ²⁻ unamended	<i>Proteobacteria</i>	<i>Epsilonproteobacteria</i>	<i>Campylobacterales</i>	<i>Helicobacteraceae</i>	<i>Sulfuricurvum</i>	
	SO ₄ ²⁻ unamended	<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfovibrionales</i>	<i>Desulfovibrionaceae</i>	<i>Desulfovibrio</i>	
	SO ₄ ²⁻ unamended	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Eubacteriaceae</i>	<i>A. malicum HAAP-1</i>	
	SO ₄ ²⁻ amended	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Unclassified</i>	
	SO ₄ ²⁻ amended	<i>Proteobacteria</i>	<i>Epsilonproteobacteria</i>	<i>Campylobacterales</i>	<i>Helicobacteraceae</i>	<i>Sulfuricurvum</i>	
	SO ₄ ²⁻ amended	<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfovibrionales</i>	<i>Desulfovibrionaceae</i>	<i>Desulfovibrio</i>	
	SO ₄ ²⁻ amended	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Eubacteriaceae</i>	<i>Acetobacterium</i>	
	SO ₄ ²⁻ amended	<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfuromonadales</i>	<i>Geobacteraceae</i>	<i>Geobacter</i>	
	SO ₄ ²⁻ amended	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Eubacteriaceae</i>	<i>A. malicum HAAP-1</i>	
Pristine Soil SIP study	Oxygen depleted	<i>Bacteroidetes</i>	-	<i>Sphingobacteriales</i>	-	-	(Jayamani et al. 2010)
		<i>Acidobacteria</i>	<i>Acidobacteria Gp4</i>	-	-	<i>Gp4</i>	
Ovine Rumen	Low N ₂ media	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Coriobacteriales</i>	<i>Coriobacteriaceae</i>	<i>Olsenella</i>	(Eaton et al. 2011)
	Low N ₂ media	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Actinomycetaceae</i>	<i>Actinomyces</i>	
	Low N ₂ media	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Lactobacillaceae</i>	<i>Lactobacillus</i>	
	Low N ₂ media	<i>Eurarchaeota</i>					
	Methanogenic	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiales Incertae Sedis XI</i>	<i>Sporanaerobacter</i>	
	Methanogenic	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Streptococcus</i>	
Ovine Rumen	Methanogenic	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Coriobacteriales</i>	<i>Coriobacteriaceae</i>	<i>Olsenella</i>	
		<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Prevotellaceae</i>	<i>Prevotella</i>	
		<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>		
		<i>Bacteroidetes</i>					
		<i>Firmicutes</i>					
		<i>Bacteroidetes</i>					
		<i>Firmicutes</i>					
		<i>TM7</i>					

3.0. A Comparative Study of Microbial Communities in Four Soil Slurries Capable of RDX Degradation using Illumina Sequencing

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Abstract

The nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has contaminated many military sites. Recently, attempts to remediate these sites have focused on biostimulation to promote RDX biodegradation. Although many RDX degrading isolates have been obtained in the laboratory, little is known about the potential of microorganisms to degrade this chemical while existing in a soil community. The current study examined and compared the RDX degrading communities in four soil slurries to elucidate the potential of natural systems to degrade this chemical. These soils were selected as they had no previous exposure to RDX, therefore their microbial communities offered an excellent baseline to determine changes following RDX degradation. High throughput sequencing was used to determine which phylotypes experienced an increase in relative abundance following RDX degradation. For this, total genomic DNA was sequenced from 1) the initial soil, 2) soil slurry microcosms following RDX degradation and 3) control soil slurry microcosms without RDX addition.

The sequencing data provided valuable information on which phylotypes increased in abundance following RDX degradation compared to control microcosms. The most notable trend was the increase in abundance of *Brevundimonas* and/or unclassified *Bacillaceae* 1 in the four soils studied. Although isolates of the family *Bacillaceae* 1 have previously been linked to RDX degradation, isolates of the genus *Brevundimonas* have not been previously associated with RDX degradation. Overall, the data suggest these two phylotypes have key roles in RDX degradation in soil communities.

3.1. Introduction

The manufacturing, transport and use of the nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has resulted in soil, groundwater and sediment contamination at many military sites. RDX has moderate solubility, low sorption and low vapor pressure resulting in significant mobility in groundwater and sediments. Due to neurotoxic effects, the potential carcinogenic nature of RDX and widespread RDX contamination, the US EPA has issued a life-time drinking water health advisory level of 2 µg/L RDX. Although RDX was initially thought to be recalcitrant, the chemical has been shown amenable to biodegradation under both aerobic and anaerobic conditions. Thus, bioremediation through the use of a carbon source has been considered a viable option to treat RDX contaminated media (Hatzinger and Lippincott 2012; Michalsen et al. In press).

The addition of a carbon source to environmental samples often results in oxygen depletion and the establishment of reducing conditions. Several bacteria have been isolated with the ability to degrade RDX under anaerobic conditions. These microorganisms classify within the phyla *Firmicutes*, *Fusobacteria* and *Proteobacteria*. The first anaerobic RDX degrading isolate obtained, *Clostridia bifermentans*, classified with the *Firmicutes*. Other *Clostridia* isolates have also been reported with RDX degrading abilities, including, for example, *Clostridia* sp. EDB2 and *Clostridia acetobutylicum*. Other RDX degrading bacteria belonging to the *Firmicutes* include two *Acetobacterium* spp. (Sherburne et al. 2005), two *Bacillus* spp. and a *Desulfotobacterium* strain. *Fusobacteria* sp. HAQ-EB21 is the only anaerobic RDX degrading isolate from the phylum *Fusobacteria*.

The *Proteobacteria*, particularly the classes *Gamma* and *Deltaproteobacteria*, contain diverse anaerobic RDX degrading isolates. In the *Gammaproteobacteria*, RDX degrading bacteria have been isolated from the families *Enterobacteriaceae*, *Pseudomonadaceae*, *Shewanellaceae* and *Xanthomonadaceae* (Binks et al. 1995). Isolates belonging to *Cystobacteraceae*, *Geobacteraceae* (Kwon and Finneran 2008a) or *Desulfovibrionaceae* (Arnett and Adrian 2009; Boopathy et al. 1998; Zhao et al. 2004b) within the *Deltaproteobacteria* are also able to degrade RDX.

Although much is known about pure cultures able to degrade RDX in the laboratory, very little is known about the microorganisms responsible for RDX degradation in mixed communities or at contaminated sites. Researchers have reported the microorganisms detected in

field samples actively degrading RDX were generally not closely related to previously reported RDX degrading isolates (Fuller and Steffan 2008). It is now widely recognized that only a small fraction of microorganisms can be isolated and cultivated in the laboratory (Amann et al. 1995), therefore, it is likely that many RDX degraders have yet to be identified. This information is important because if several key species were consistently linked to effective RDX degradation in mixed communities, their presence could provide strong evidence of RDX natural attenuation.

In the current study, high throughput sequencing was used to compare four soil slurry communities to determine which phylotypes were enriched following RDX degradation. The overall aim was to ascertain if similarities in enrichment patterns could be observed across these four soils. This enrichment is of interest because it indicates these microorganisms are increasing in abundance because of RDX degradation and are therefore likely benefiting from the degradation process (e.g. using this chemical as a carbon, energy or nitrogen source). Such information is valuable because the enriched phylotypes could then be used as biomarkers for RDX degradation potential. The current study focuses on the phylotypes that were enriched following RDX degradation in four soil communities in the presence of the carbon source glucose.

3.2. Methods

3.2.1. Chemicals

RDX dissolved in acetonitrile were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). High performance liquid chromatography (HPLC) grade acetonitrile (≥ 99.8 % purity) was purchased from EMD Chemicals Inc. (New Jersey, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher BioReagent (New Jersey, USA), or Invitrogen (Carlsbad, CA, USA) unless otherwise stated.

3.2.2. Experimental Design

Four agricultural soils (referred to as Soil 9-12, Table 1) were utilized in these experiments and, to our knowledge, these soils have not previously been exposed to RDX. The soil slurry microcosms for screening RDX degradation were established as previously described, with only slight modifications. Briefly, for each soil enrichment, 60 mL serum bottles contained 1 g soil (wet weight), 20 mg L⁻¹ RDX (dissolved in acetonitrile), 202 mg L⁻¹ glucose in 4 mL of a

minimal salts media (MSM) . From the twelve soils screened, four (Soils 9, 10, 11, 12) were selected for additional studies based on their more rapid RDX degradation rates.

For the high throughput sequencing study, the microcosms were prepared with 1 g soil (wet weight), 1 mL of inocula (a mixture of suspended soil and water) from the corresponding RDX screening microcosm, 20 mg L⁻¹ unlabeled or ring-labeled RDX (¹³C₃, 99%; ¹⁵N₃, 98%) (dissolved in acetonitrile), 202 mg L⁻¹ glucose and 4 mL of a minimal salts media (MSM). The inocula were used to increase the speed of RDX degradation in the live microcosms. Four sample microcosms (hereafter referred to as “Microcosm 1, 2, 3 or 4”) and triplicate abiotic controls (autoclaved) were amended with RDX. Microcosms 1 and 2 received ring-labeled RDX and Microcosms 3 and 4 received unlabeled RDX (a stable isotope probing experiment was planned with these samples, however, time did not permit the completion of these experiments). Another amendment of 20 mg L⁻¹ of RDX was added to the sample microcosms following the depletion of the first amendment. Specifically, RDX was added on day 32 (Soil 9), day 16 (Soil 10), day 27 (Soil 11) and day 20 (Soil 12). The study also included control microcosms treated in the same manner as the sample microcosms, except RDX was not added (acetonitrile was added, hereafter referred to as “No RDX controls”).

All microcosms were sealed with rubber stoppers and aluminum seals and were covered with aluminum foil to prevent photodegradation. The microcosms were incubated between 3 to 7 weeks in the dark at room temperature (~20 °C) without shaking while being monitored for RDX degradation.

3.2.3. RDX Extraction and Analysis

RDX concentrations were determined using HPLC as previously described (Thompson et al. 2005) with modifications. For this, 0.5 mL (extracted using a 1 mL BD syringe with a 21 gauge needle) was placed in a 1.7 mL sterile microcentrifuge tube. An equal volume of acetonitrile was added and the microcentrifuge tubes were shaken for 2 hours at room temperature to extract RDX. The samples were then centrifuged for 5 minutes at 10,000 x g and the supernatant was filtered using acetonitrile wetted filters (PVDF, 0.22 µm, Whatman) into HPLC amber vials (Sigma-Aldrich, St. Louis, MO, USA). External standards for the calibration curve were prepared with a dilution factor of 2 to account for the sample dilution at the liquid-liquid extraction step. HPLC analysis involved a Perkin Elmer (PE) series 200 autosampler; PE binary

LC Pump 250; PE diode array detector 235C, at wavelength 255nm; Supelco C18 (25 cm X 4.6 mm, 5 μ m) column; and isocratic conditions (40% acetonitrile and 60% 0.1% H₃PO₄ acidified deionized water) at a flow rate of 1 mL min⁻¹.

3.2.4. DNA Extraction

DNA was extracted from all sixteen microcosms (Microcosms 1, 2, 3, 4 and No RDX controls for all four soils) following the degradation of the second amendment of RDX (day 43 for Soil 9, day 23 for Soil 10, day 39 for Soil 11 and day 28 for soil 12). Further, DNA was extracted from each soil prior to any manipulation (hereafter called “No RDX, time 0”). The total genomic DNA from all four soils was extracted to establish the baseline microbial community. All DNA extractions used the Power Soil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA).

3.2.5. Amplicon Sequencing and Data Analysis

DNA extracted from all sixteen microcosms were submitted for amplicon sequencing at Michigan State University’s Research Technology and Support Facility (RTSF). Total genomic DNA extracted from all four of the initial soils (no RDX, time 0) were also amplicon sequenced. Amplification of the V4 region and paired-end high throughput amplicon sequencing on the Illumina MiSeq platform at RTSF was conducted using a procedure previously described (Caporaso et al. 2012; Caporaso et al. 2011).

Sequencing data obtained from the MiSeq platform Laboratory Information Management System (LIMS) was analyzed using Mothur v.1.33.2 using the MiSeq standard operating procedure (Kozich et al. 2013). The sequence data in the fastq format were processed using Mothur to remove the barcodes and these were then aligned to form contiguous sequences. The data was checked for sequencing errors and read length. The sequences were then aligned and checked for chimeras using UCHIME in Mothur (Edgar et al. 2011). Following which, the sequences were classified into OTU’s and phylotypes using the Ribosomal Database Project dataset within Mothur. The sequence data was then imported into Microsoft Excel for further analysis. Illumina sequencing data were deposited to the NCBI Sequence Read Archive under Bioproject Number PRJNA263419.

3.3. Results and Discussion

3.3.1. RDX Degradation

All four agricultural soils slurries illustrated RDX degradation. The first amendment of RDX was degraded in 15 to 31 days and the second amendment was degraded following an additional 7 to 12 days. Soils 10 and 12 illustrated the fastest RDX removal. No significant degradation of RDX was observed in the killed control microcosms. Oxygen concentrations were not monitored in these microcosms, however, preliminary experiments with Soils 9-12 involved opening the microcosms daily and these did not result in any RDX removal. Thus, RDX degradation in these soils was likely limited to oxygen depleted conditions.

3.3.2. Comparison at Phyla Level

Total genomic DNA from six samples (initial soil, no RDX controls and four microcosms) for all four soils were submitted for amplicon sequencing. An average of 118,582 reads was obtained per sample. On average, 27% of the sequences did not align properly into contiguous sequences resulting in read lengths >275 bp and these were dropped from the analysis. Another 10% of the sequences were identified as chimeric or as non-bacterial (mitochondrial/chloroplast) by the Bayesian classifier within Mothur.

Phylotype classification and sequence abundance data were used to assess the microbial communities in each of the four soils (Figure 1). The relative abundance of each phyla for all four initial soils (No RDX, time 0) were remarkably similar. That is, the phylotypes classified into *Firmicutes*, *Proteobacteria*, *Actinobacteria* (the three most dominant), *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi* and *Gemmatimonadetes*. In contrast, for all four soils, in both the RDX amended microcosms (Microcosms 1, 2, 3 & 4) and in the microcosms not amended with RDX (No RDX controls), the communities were primarily composed of *Proteobacteria* (particularly soils 10 and 11) and *Firmicutes* (particularly soils 9 and 12). This increase in *Firmicutes* is likely a result of oxygen depletion in these samples (the microcosms were sealed for more than 20 days). For the RDX amended samples, these results are consistent with the phyla containing the majority of anaerobic RDX degrading isolates (these belong primarily to the phyla *Firmicutes* and *Proteobacteria*, Table 2). In addition, other mixed cultures illustrating RDX degradation have been dominated by these two phyla .

At the phyla level, for soils 9 and 12, the classifications were similar between the microcosms amended with RDX and those not amended with RDX. For soils 10 and 11, the abundance of *Proteobacteria* increased when RDX was amended. Although phyla level classifications provide a useful baseline to compare these communities, comparisons at the phylotype level are more informative and will be discussed below.

3.3.3. Microbial Community Analysis

Rarefaction curves were generated for the initial soils (No RDX, time 0), for the microcosms amended with RDX (Microcosms 1, 2, 3 & 4) and for the microcosms without RDX (No RDX controls) (Figure 2a). The curves are all similar except for three (soils 10, 11 and 12) of the four initial soils, which illustrated a much steeper gradient, indicating incomplete sequence coverage for these samples. The curves for the other samples started to plateau, suggesting more complete sequence coverage.

Principal component analysis was performed on the sequencing data (Figure 2b). Clustering occurred for each soil in the four samples amended with RDX (Microcosms 1, 2, 3 & 4). The clusters for soils 10 and 11 were close together, as were the clusters for soils 9 and 12. Separate clusters were also formed for each of the four time 0 soil samples (No RDX, time 0) and for the four soil microcosms without RDX addition (No RDX controls).

To more precisely compare the relative abundance of phylotypes across treatments, the most abundant phylotypes (>1% relative abundance) for each microbial community were determined. The most abundant phylotypes were similar across all four initial soils (No RDX, time 0) (Figure 3a). The majority of the abundant phylotypes classified within the *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, with only one in each of the *Chloroflexi*, *Verrucomicrobia*, *Gemmatimonadetes* and *Bacteroidetes*. Each of the four soils contained a large relative abundance of unclassified *Bacteria*. In general, the most abundant phylotypes in all four soils included unclassified *Bacillales*, unclassified *Sphingomonadaceae*, *Arthrobacter*, unclassified *Actinomycetales*, unclassified *Actinobacteria*, *Acidobacteria Gp1* and *Gp 3* and unclassified *Chitinophagaceae*. The most abundant phylotypes in the microcosms not amended with RDX (No RDX controls) were different (Figure 3b). These phylotypes only classified within the *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. The most abundant phylotypes were primarily *Firmicutes*, including *Clostridium XIVa*, *Tissierella*, *Sedimentibacter*

and unclassified *Clostridiaceae 1*. In addition, the phylotypes *Rhodococcus* and unclassified *Chitinophagaceae* were abundant in three of the four soils. Not surprisingly, these data clearly indicate that the incubation conditions (sealed bottles with glucose and a minimal salts media) completely changed the microbial community structure. Interestingly, the microbial community that developed following incubation was similar between soils.

The most abundant phylotypes were also determined for the microcosms amended with RDX (Figure 4). Similar to the data discussed above (No RDX controls), the abundant phylotypes classified only within the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (only for soils 10 and 11). Again, the majority of abundant phylotypes classified within the *Firmicutes*. Similar phylotypes were enriched across all four soils. For the *Firmicutes* this included *Sedimentibacter*, *Tissierella*, unclassified *Clostridiaceae 1* and unclassified *Bacillaceae 1*. Other common phylotypes included *Brevundimonas* (soils 10, 11 and 12), *Rhodococcus* and unclassified *Bacteria* (all four soils). In general, the relative abundance values were similar between the four microcosms.

3.3.4. Phylotypes Increasing in Abundance Following RDX Degradation

The communities were further examined to ascertain which phylotypes increased in abundance following RDX degradation compared to the initial soils and the microcosms without RDX. These data indicate which phylotypes are gaining a benefit from RDX degradation (perhaps as an energy, carbon or nitrogen source). For each soil, this involved the selection of the eight most abundant phylotypes in the microbial communities following RDX degradation. Following this, for each soil, the relative abundance of these eight phylotypes was determined in the initial soils and in the microcosms not amended with RDX. The comparison of these data for each soil has been summarized (Figure 5).

The data analysis produced two interesting common trends between the four soils. The phylotypes *Brevundimonas* (*Proteobacteria*, *Alphaproteobacteria*, *Caulobacterales*, *Caulobacteraceae*) and unclassified *Bacillaceae 1* (*Firmicutes*, *Bacilli*, *Bacillales*) were both dominant following RDX degradation compared to the controls (initial soil and no RDX controls) in at least three of the four soils. Specifically, *Brevundimonas* illustrated a clear increase in relative abundance following RDX degradation in all four microcosms for soils 10, 11 and 12, compared to the controls. The relative abundance varied between 34-52%, 47-58%

and 10-15% for soils 10, 11 and 12, respectively. The same trend occurred for unclassified *Bacillaceae 1* for soils 9, 11 and 12. For this phylotype, the relative abundance varied between 12-25%, 15-22% and 30-46% for soils 9, 11 and 12, respectively.

The family *Bacillaceae 1* contains 36 genera, any of which could have been involved in RDX degradation in the current study. *Bacillus* is the only genus within the family previously linked to RDX degradation. Members of this family were also dominant among the RDX degrading microbial community from a saturated contaminated surface soil and have previously been reported to be involved in PCB (Bertin et al. 2011) and PAH (Wu et al. 2008) degradation.

The phylotype *Brevundimonas* classifies within the *Alphaproteobacteria*. Although many known anaerobic RDX degraders belong to the phylum *Proteobacteria*, only one RDX isolate (*Methylobacterium* sp.) belongs to the *Alphaproteobacteria*. Therefore, the current study has provided new insights into the importance of this phylotype for RDX degradation. In other research, *Brevundimonas* was present in RDX contaminated groundwater although at a lower level (1 16S rRNA clone from 120 sequences). *Brevundimonas* was also involved in the degradation of an organophosphorus insecticide (Deshpande et al. 2004) and cellulose (Konno et al. 2006). They have also been associated with direct petroleum hydrocarbons degradation (Chaineau et al. 1999; Chang et al. 2007) and with the root-associated bacterial communities of plants involved in phytoremediation (Phillips et al. 2008).

The phylotypes *Rhodococcus*, *Tisserella* (soil 9), unclassified *Erysipelotrichaceae* (soil 10), unclassified *Alcaligenaceae* (soil 11) and *Flavobacterium* (soil 12) were also more abundant in the RDX treated samples in comparison to the no RDX controls, however, their relative abundance values were lower than *Brevundimonas* or unclassified *Bacillaceae 1* (Figure 5).

3.4. Conclusions

The high throughput sequencing data provided valuable information on which phylotypes increased in abundance following RDX degradation compared to the initial soil and the microcosms not amended with RDX. The most notable trend was the common increase in abundance of *Brevundimonas* and/or unclassified *Bacillaceae 1* in the four soils studied. The data indicate these two phylotypes are benefiting from RDX degradation under these conditions. This information is important because the presence of these phylotypes in soil and sediment samples from contaminated sites could provide a line of evidence for the use of natural

attenuation to remediate such sites. Future research will determine the abundance of these phylotypes at RDX contaminated sites.

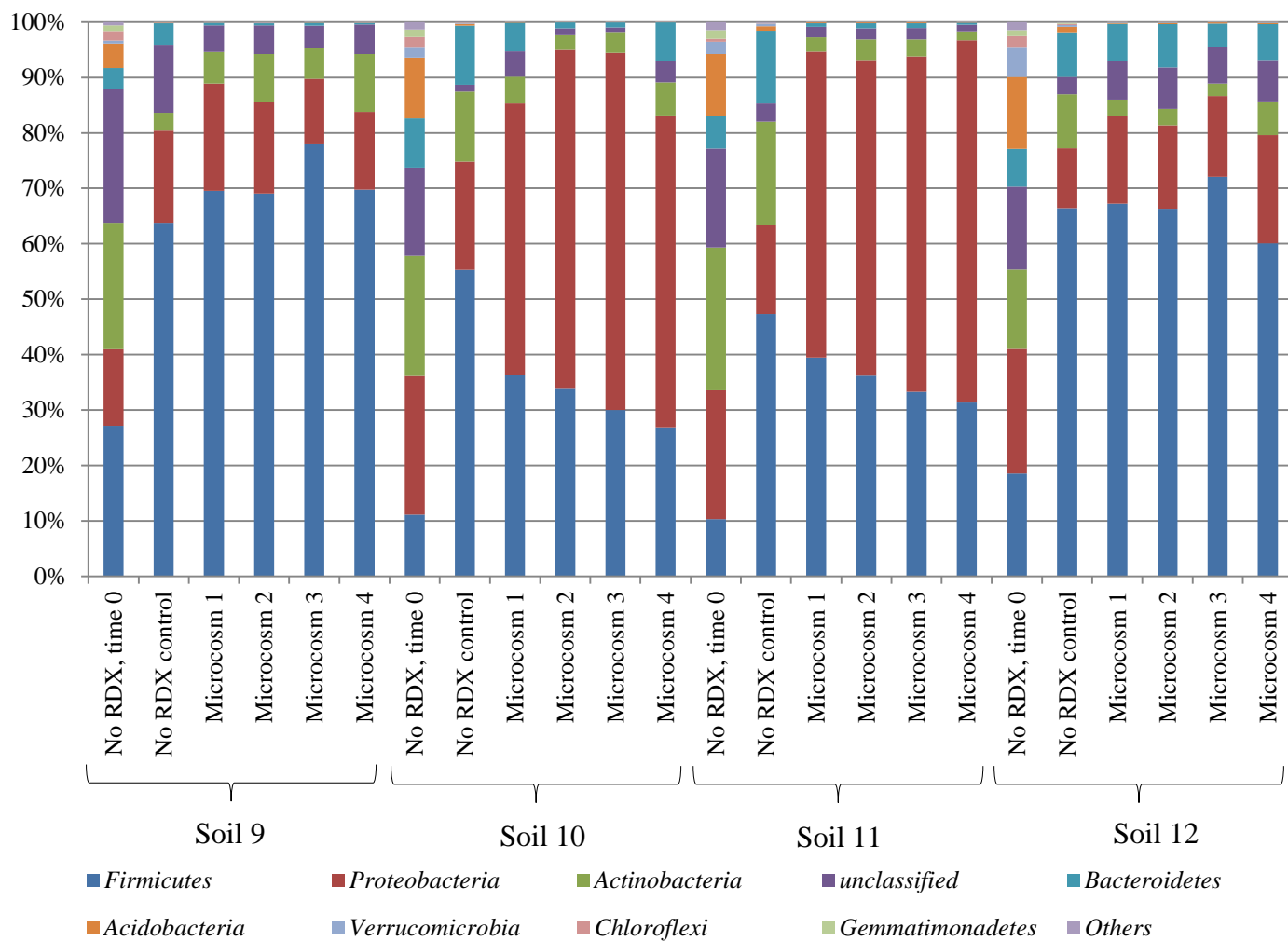


Figure 3.1. Phyla level comparison between the four soils in 1) the initial soils (No RDX, time 0), 2) four microcosms and 3) microcosms without RDX addition (No RDX controls).

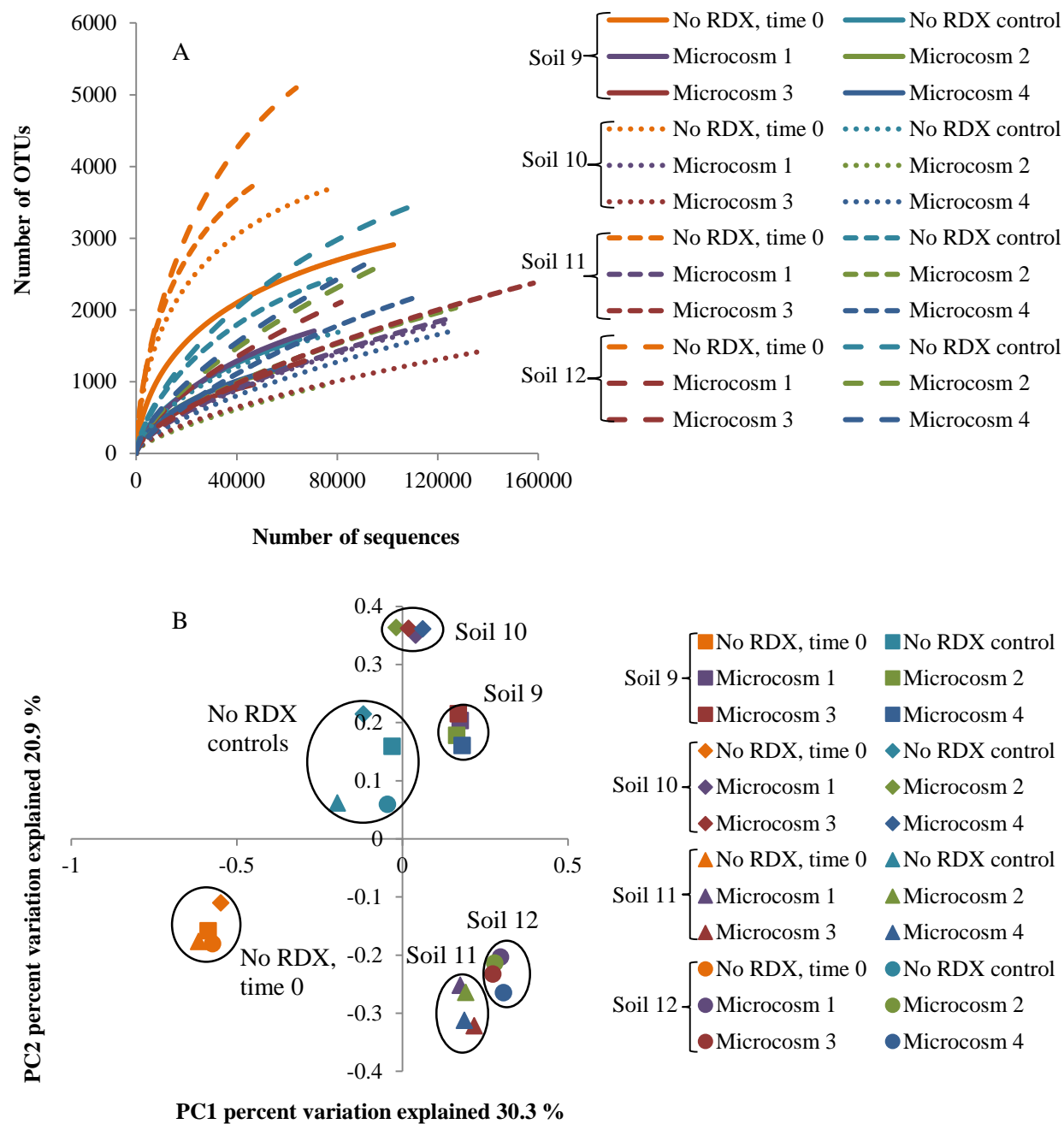


Figure 3.2. Rarefaction curves (a) and Principal Component Analysis (b) of sequencing data generated from total DNA extracts of labeled and unlabeled microcosms from all four soils.

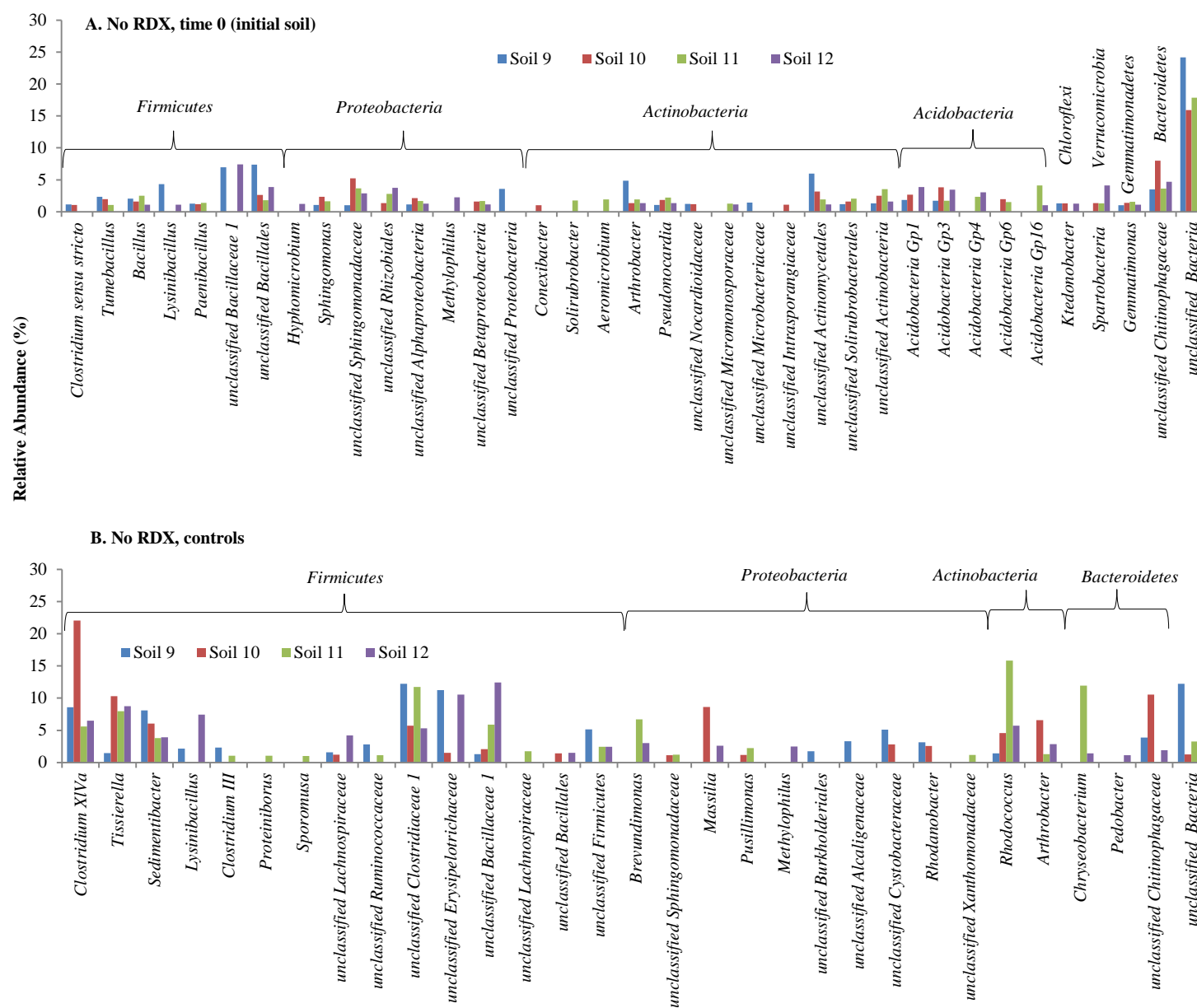


Figure 3.3. A comparison of the relative abundance (%) of the most common phylotypes (>1% , at the lowest classification level) in all four soils in the no RDX samples, including the initial soils (No RDX, time 0) (A) and the microcosms without RDX (No RDX controls) (B).

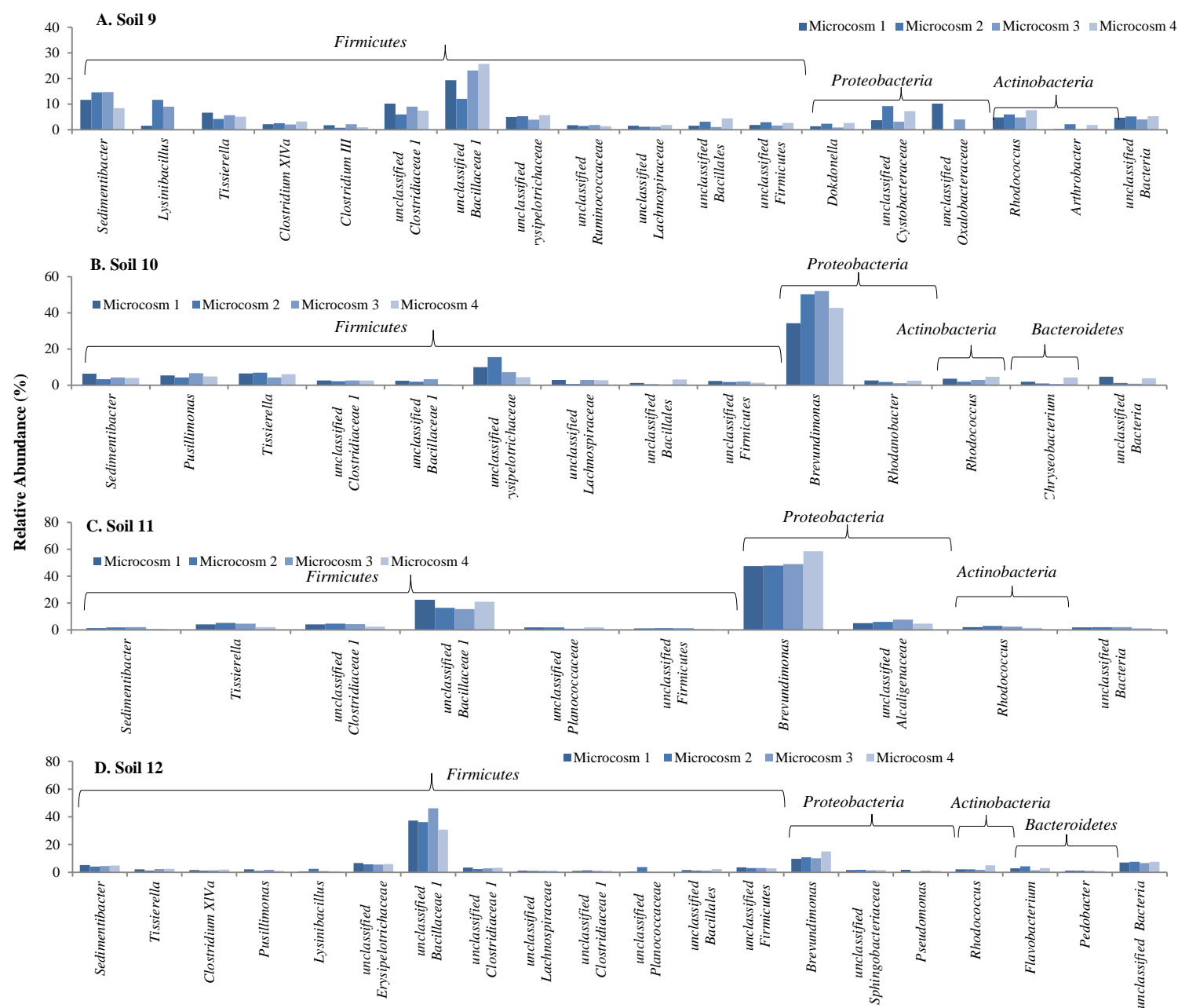


Figure 3.4. A comparison of the relative abundance (%) of the most common phylotypes (>1%, at the lowest classification level) in all four soils (A-D) following RDX degradation.

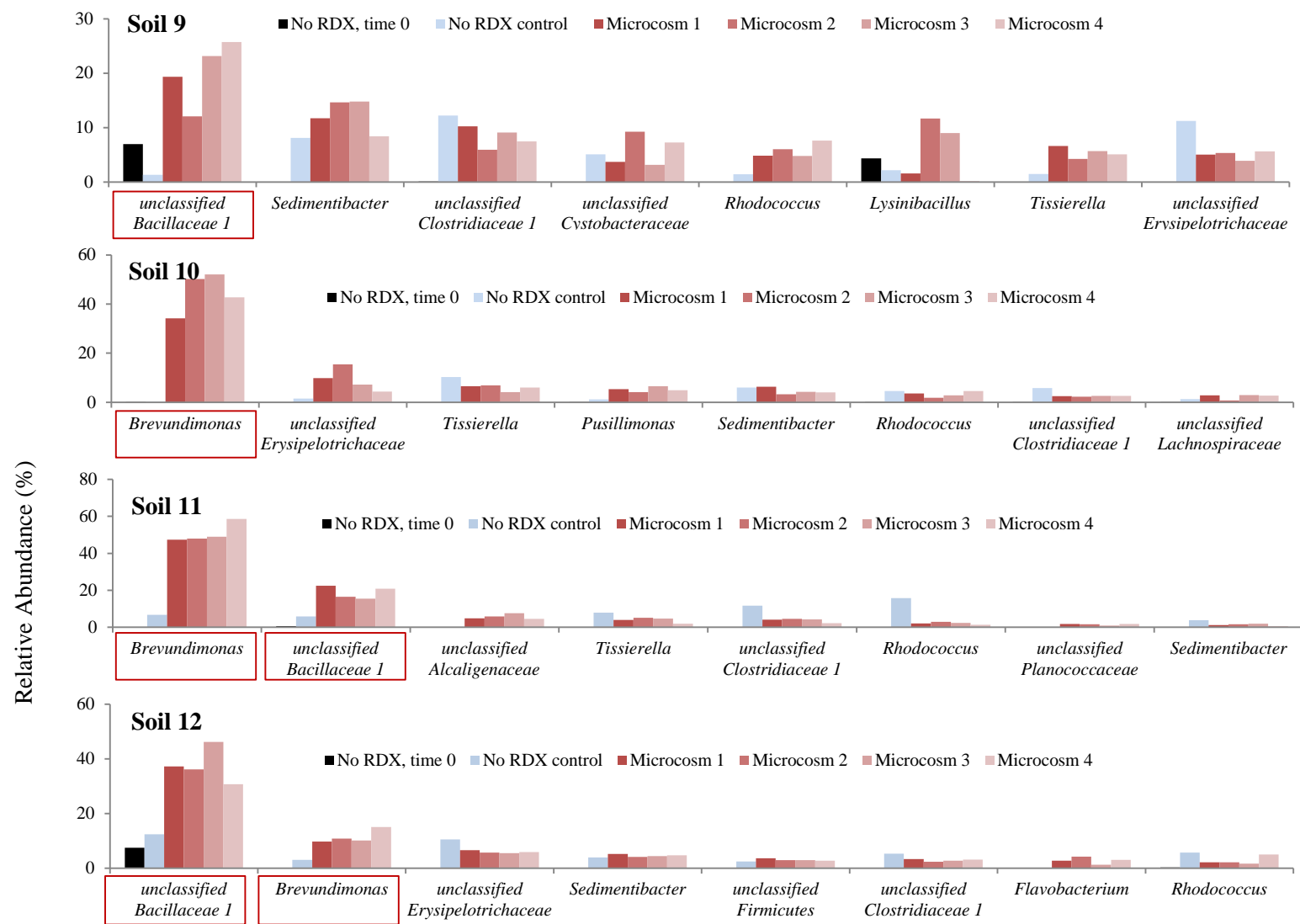


Figure 3.5. The relative abundance (%) of the eight most abundant phylotypes in the four RDX amended agricultural soils compared to the RDX controls (initial soil and no RDX controls). Boxed phylotypes illustrate a greater abundance in RDX amended samples compared to the RDX controls for at least three soils. The phylotypes are at genus level, unless unclassified.

Table 3.1. The properties of the four agricultural soils used in this study.

Soil	Crop	Organic Matter %	Sand %	Silt %	Clay %	Textural Classification	Soil pH	Calcium mg/kg	Magnesium mg/kg	Potassium mg/kg	Phosphorus mg/kg	CEC meq/100 g	OM Level
9	Corn	1.2	70	24	6	Sandy Loam	4.9	489	68	151	46	5	High
10	Corn	1	76	22	2	Loamy Sand	5.5	787	42	108	79	5	Low
11	Alfalfa	1.5	80	20	0	Loamy Sand	6.4	811	74	173	106	4.6	Low
12	Corn	2.2	86	14	0	Sand	6.1	1312	242	139	268	8.6	Low

Table 3.2. The classification of anaerobic RDX degrading isolates from previous studies.

Class	Order	Family	Full name	Reference(s)
Phylum Firmicutes				
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Clostridium bifermentans</i>	(Regan and Crawford 1994)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Clostridium bifermentans</i> (HAW-1, HAW-G3, HAW-G4, HAW- E3 and, HAW-EC1)	(Zhao et al. 2003)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Clostridium sp. HAW-EB17</i>	(Zhao et al. 2004c)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Clostridium sp. EDB2</i>	(Bhushan et al. 2004)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Clostridium acetobutylicum</i>	(Zhang and Hughes 2003)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Acetobacterium malicum sp.</i> HAAP-1	(Adrian and Arnett 2004; Zhang and Hughes 2003)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Clostridiaceae</i>	<i>Acetobacterium paludosum</i>	(Sherburne et al. 2005)
<i>Clostridia</i>	<i>Clostridiales</i>	<i>Peptococcaceae</i>	<i>Desulfotobacterium sp.</i>	(Kwon and Finneran 2008b)
<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus sp. HPB2 and HPB3</i>	(Singh et al. 2009)
Phylum Fusobacteria				
<i>Fusobacteria</i>	<i>Fusobacteriales</i>	<i>Fusobacteriaceae</i>	<i>Fusobacteria HAW-21</i>	(Zhao et al. 2004b)
Phylum Proteobacteria				
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Serratia sp.</i>	(Young et al. 1997)
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Enterobacter sp.</i>	(Kitts et al. 2000; Pudge et al. 2003)
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Citrobacter sp.</i>	
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Morganella sp.</i>	
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Klebsiella sp.</i>	(Zhao et al. 2002)
<i>Gammaproteobacteria</i>	<i>Enteriobacteriales</i>	<i>Enteriobacteriaceae</i>	<i>Providencia sp.</i>	
<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas sp.</i>	(Singh et al. 2009)
<i>Gammaproteobacteria</i>	<i>Alteromonadales</i>	<i>Shewanellaceae</i>	<i>Shewanella sp.</i>	(Zhao et al. 2005; Zhao et al. 2006; Zhao et al. 2004b)
<i>Gammaproteobacteria</i>	<i>Xanthomonadales</i>	<i>Xanthomonadaceae</i>	<i>Strenophomonas sp.</i>	(Binks et al. 1995)
<i>Deltaproteobacteria</i>	<i>Desulfovibrionales</i>	<i>Desulfovibrionaceae</i>	<i>Desulfovibrio sp.</i>	(Arnett and Adrian 2009; Boopathy et al. 1998; Zhao et al. 2004b)
<i>Deltaproteobacteria</i>	<i>Desulfuromonadales</i>	<i>Geobacteraceae</i>	<i>Geobacter sp.</i>	(Kwon and Finneran 2008a)
<i>Deltaproteobacteria</i>	<i>Myxococcales</i>	<i>Cystobacteraceae</i>	<i>Anaeromyxobacter sp.</i>	(Kwon and Finneran 2008b)

4.0. Stable Isotope Probing Reveals the Importance of *Comamonas* and *Pseudomonadaceae* in RDX Degradation in Samples from a Navy Detonation Site

This chapter was published (Jayamani and Cupples 2015b) as follows:

Jayamani I, Cupples AM (2015b) Stable isotope probing reveals the importance of *Comamonas* and *Pseudomonadaceae* in RDX degradation in samples from a Navy detonation site.

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Abstract

This study investigated the microorganisms involved in RDX degradation from a detonation area at a Navy Base. Using Illumina sequencing, microbial communities were compared between the initial sample, samples following RDX degradation and controls not amended with RDX to determine which phylotypes increased in abundance following RDX degradation. The effect of glucose on these communities was also examined. In addition, stable isotope probing (SIP) using labeled ($^{13}\text{C}_3$, $^{15}\text{N}_3$ – ring) RDX was performed.

Illumina sequencing revealed several phylotypes were more abundant following RDX degradation compared to the initial soil and the no RDX controls. For the glucose amended samples, this trend was strong for an unclassified *Pseudomonadaceae* phylotype and for *Comamonas*. Without glucose, *Acinetobacter* exhibited the greatest increase following RDX degradation compared to the initial soil and no RDX controls. *Rhodococcus*, a known RDX degrader, also increased in abundance following RDX degradation.

For the SIP study, unclassified *Pseudomonadaceae* was the most abundant phylotype in the heavy fractions in both the presence and absence of glucose. In the glucose amended heavy fractions, the 16S rRNA genes of *Comamonas* and *Anaeromyxobacter* were also present. Without glucose, the heavy fractions also contained the 16S rRNA genes of *Azohydromonas* and *Rhodococcus*. However, all four phylotypes were present at a much lower level compared to unclassified *Pseudomonadaceae*. Overall, these data indicate unclassified *Pseudomonadaceae* were primarily responsible for label uptake in both treatments. This study indicates, for the first time, the importance of *Comamonas* for RDX removal.

4.1. Introduction

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is an explosive that has caused widespread soil and water contamination at many military sites in the US and worldwide (EPA 2014). Such contamination has been associated with manufacturing and load-assemble-package processes performed during or after World War II and the Korean Conflict. Remediation of these sites has been initiated since the early 1980s, however many still have groundwater contaminated with nitroaromatics. The U.S. EPA has established a health advisory level in drinking water for RDX (2 µg/L), indicating the potential threat to humans (EPA 2014). RDX is susceptible to aerobic or anaerobic biodegradation (Adrian and Arnett 2004; Adrian and Chow 2001; Beller 2002; Kitts et al. 1994; Kitts et al. 2000; Young et al. 1997; Zhao et al. 2004a), therefore bioremediation offers a plausible approach for site clean-up. Anaerobic RDX degradation can occur via hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (McCormick et al. 1981), forming methanol and formaldehyde. Denitration has also been reported under anaerobic conditions, involving ring cleavage and methylene denitramine (MEDINA) formation (Zhao et al. 2002). Under aerobic conditions, denitration appears to be a common pathway (Cupples 2013). RDX denitration has been coupled with ring cleavage generating 4-nitro-2,4-diazabutanal (NDAB) as a product (Bernstein and Ronen 2012). MEDINA has also been observed as a ring cleavage product (Halasz et al. 2010). In all, known RDX degrading aerobic or anaerobic isolates classify within the phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria* (*Alpha*-, *Gamma*- and *Deltaproteobacteria*) and *Fusobacteria* (Cupples 2013). However, researchers have reported the microorganisms detected in field samples actively degrading RDX were generally not closely related to previously reported RDX degrading bacterial strains (Fuller and Steffan 2008). The identification of RDX degraders at these sites is important for 1) determining if natural attenuation is a feasible approach across sites and 2) monitoring the populations of the microorganisms involved.

Although much is known about the pure cultures able to degrade RDX in the laboratory, very little is known about the microorganisms responsible for RDX degradation at contaminated sites. One group concluded that previously reported RDX degrading bacteria did not capture the microbial diversity associated with RDX bioremediation in groundwater, especially under typical biostimulation approaches (Fuller et al. 2010). Indeed, it is now widely recognized that only a

small percentage of microorganisms can be isolated and cultivated in the laboratory (Amann et al. 1995), therefore, it is likely that in situ RDX degraders have yet to be identified. However, recent advances in molecular biology have enabled a wealth of information concerning a range of microbial processes. A number of molecular approaches have been used to investigate RDX degraders in mixed communities, such as terminal restriction fragment length polymorphism (TRFLP), Sanger sequencing (Fuller et al. 2010; Kwon and Finneran 2010; Kwon et al. 2011; Ronen et al. 2008) and stable isotope probing (SIP) (Andeer et al. 2013; Cho et al. 2013; Roh et al. 2009). SIP is a culture independent method that targets only active organisms and involves sample exposure to a labeled substrate, incubation, nucleic acid extraction, ultracentrifugation to separate the labeled nucleic acid from the unlabeled background nucleic acid, and finally molecular analysis to identify the organism(s) responsible for label uptake. The method is advantageous because there is no requirement to work with pure cultures and so SIP can identify the microorganisms involved in label uptake in mixed cultures, e.g. soil samples. The method has been particularly valuable for detecting microorganisms involved in contaminant degradation (Sun and Cupples 2012; Sun et al. 2012; Sun et al. 2010; Xie et al. 2010; Xie et al. 2011).

To date, for RDX degradation studies, SIP has only been combined with TRFLP and/or Sanger sequencing. These approaches have their limitations because it is not always possible to sequence enough clones to identify the phylotypes involved in label uptake. In this study, we advance this methodology by combining SIP with high throughput sequencing to investigate the microorganisms responsible for label uptake from RDX. This is the first report of using both methods to examine RDX degraders.

Here, the objective was to identify the microorganisms involved in RDX degradation in soils from a detonation area at a Navy Base. The research also explores the effect of glucose on RDX degradation and the microorganisms involved. RDX biodegradation and bioremediation often involves the addition of a carbon source (Michalsen et al. In press). Glucose was added in this study, based on previous research indicating enhanced RDX degradation in other soil samples when this substrate was added (Jayamani et al. 2013). The microbial communities were investigated both in the presence and in the absence of glucose. The research combined SIP and high throughput sequencing to provide two layers of data. Firstly, phylotypes illustrating an increase in relative abundance following RDX degradation compared to controls (initial soil and samples with no RDX added) were identified with Illumina sequencing. Secondly, DNA based

SIP (^{15}N and ^{13}C RDX) was used to identify which microorganisms were involved in label uptake from RDX. The wealth of information provided by coupling high throughput sequencing with SIP has enabled the identification of novel RDX degraders and has provided data that can be used to design primers for the detection of these microorganisms at other RDX contaminated sites.

4.2. Methods

4.2.1. Chemicals

Unlabeled and ring- labeled RDX ($^{13}\text{C}_3$, 99%; $^{15}\text{N}_3$, 98%) in acetonitrile (1,000 mg/L) were purchased from Cambridge Isotope Laboratories (Andover, MA). Other reagents were purchased from Sigma Aldrich® Corp. (St. Louis, MO) or Thermo Fisher Scientific Inc. (Waltham, MA), unless otherwise stated. HPLC grade acetonitrile was purchased from EMD Millipore (Chemicals), a division of Merck KGaA (Darmstadt, Germany).

4.2.2. Experimental Setup and DNA Extraction

The soil was collected from a Navy Installation in Virginia. Soil samples were first screened for their ability to degrade RDX (no additional details on the Navy Base can be provided for publication). For this, duplicate killed controls (autoclaved) and live microcosms were prepared using 4 mL of a mineral salts media (MSM) (0.272 g KH_2PO_4 ; 0.348 g K_2PO_4 ; 0.2 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.03 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.5 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.05 mg H_3BO_3 ; 0.05 mg ZnCl_2 ; 0.03 mg CuCl_2 ; 0.01 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 0.5 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.05 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.5 mg Na_2SeO_3 per liter) (Thompson et al. 2005), 1 mL of glucose (1 g L^{-1}), 1.5 g of soil (dry weight) and 10 mg L^{-1} of unlabeled RDX in amber serum bottles. Microcosms were sealed with rubber stoppers and aluminum seals and stored in the dark ($\sim 20^\circ\text{C}$) without shaking. A summary of DNA extraction times and the amendments for each sample has been created (Table 1). For the SIP study, microcosms were prepared with or without glucose. The serum bottles (30 mL) included 2 g of soil with either 4 mL of mineral media and 1 mL of glucose (1 g L^{-1} stock solution) or 5 mL of MSM. Then, 10 mg L^{-1} of labeled ($^{13}\text{C}_3$, $^{15}\text{N}_3$ -RDX) or unlabeled RDX was added. A total of 2 killed controls (sterilized by autoclaving), 4 unlabeled controls (10 mg L^{-1} unlabeled RDX) and 2 labeled sample microcosms (10 mg L^{-1} of labeled $^{13}\text{C}_3$, $^{15}\text{N}_3$ -RDX) were prepared for each of the two treatments (with or without glucose). Two

additional controls (no RDX controls) were also included, both were not amended with RDX (acetonitrile was added, as RDX from above was dissolved in acetonitrile) and only one was amended with glucose. These controls were incubated under the same conditions as the above samples. All of the microcosms and controls were not aerated during the incubation period.

Following the degradation of the first addition of RDX (30 days), eight microcosms (replicate microcosms with labeled RDX and glucose, unlabeled RDX and glucose, labeled RDX and no glucose and unlabeled RDX and no glucose) and the two no RDX controls (with and without glucose) were sacrificed and the total genomic DNA was extracted. DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) as per manufacturer's instructions. In the remaining replicate microcosms initially amended with unlabeled RDX (with and without glucose), a second amendment of 10 mg L⁻¹ of RDX (unlabeled) was added. This was degraded under 20 days and the total genomic DNA was extracted from these samples. DNA was also extracted from the initial soil. In all, fifteen total genomic DNA extracts were submitted for Illumina sequencing (a list is provided below).

4.2.3. Analytical Methods

RDX extraction and analysis methods were modified from those previously described (Thompson et al 2005). Briefly, 200 µL from each microcosm was removed into 1.7 mL microcentrifuge tubes. RDX was extracted by adding equal volumes of acetonitrile and shaking the tubes at room temperature for 30 minutes. Following this, the tubes were centrifuged at 10,000 rpm for 5 minutes. The supernatant (350 µL) was filtered using 0.22 µm sterile filters into HPLC amber vials (Sigma). The HPLC parameters were as follows: injector volume, 20 µL; isocratic conditions (40% acetonitrile and 60% 0.01% H₃PO₄-acidified water, 1 mL/min); Perkin Elmers (PE) Series 200 autosampler; PE binary LC Pump 250; Waters UV detector; wavelength 255 nm (detection limit was 500 µg/L).

4.2.4. Isopycnic Centrifugation

Total genomic DNA extracted from four microcosms with one amendment of RDX was subject to ultracentrifugation and fractioning. This involved two microcosms amended with glucose (labeled and unlabeled RDX amended) and two not amended with glucose (labeled and unlabeled RDX amended). The extracted DNA was ultracentrifuged with cesium chloride and

tris-EDTA buffer solution. Specifically, 3 to 5 μg of DNA was added to a Tris-EDTA (pH 8.0) buffer and cesium chloride solution. The initial buoyant density (BD) of this mixture was adjusted to $\sim 1.72 \text{ g mL}^{-1}$ using a model AR200 digital refractometer (Leica Microsystems Inc.). This mixture was then loaded to a 5.1 mL Quick-Seal polyallomer tubes (1.3 x 5.1 cm, Beckman Coulter) and sealed using a tube topper (Cordless quick-seal tube topper, Beckman). The tubes were balanced in a Stepsaver 70 V6 Vertical Titanium Rotor (Thermo Scientific) and centrifuged at $178,000 \times g$ for 46 hours at 20°C in a Wx Sorvall Ultracentrifuge to obtain density gradients. The density gradients were separated into 20 fractions (each 250 μL) using gravity by displacing the samples by molecular grade water pumped by a syringe pump. Each fraction was mixed and sampled to measure their refractive index to calculate the buoyant density. The fractions were cleaned using a glycogen and ethanol precipitation to remove the cesium chloride and precipitate the DNA. They were then re-suspended in 30 μL PCR grade molecular water and stored at -20°C until further analysis. The concentration of DNA in each fraction was quantified with the Quant-iTTM dsDNA High-Sensitivity Assay Kit using the Qubit[®] 2.0 Fluorometer.

4.2.5. Illumina MiSeqTM High Throughput Amplicon Sequencing

Both total genomic DNA extracts and selected fractions were submitted for high throughput amplicon sequencing following the protocol described elsewhere (Caporaso et al. 2012; Caporaso et al. 2011) at the Research Technology Support Facility (RTSF) at Michigan State University. Fractions were selected based on the comparison of DNA concentrations over the buoyant density range in the fractions obtained from the unlabeled and labeled RDX amended microcosms. Those fractions from the labeled RDX amended microcosms illustrating a higher DNA concentration at the higher buoyant density values were selected for sequencing. These fractions were examined because the increased concentration at higher buoyant density values indicated the DNA in these fractions contained a heavy label. Thus, the phylotypes present in these fractions could be linked to RDX assimilation. Six fractions were sequenced for each of the four samples ultracentrifuged. Further, each fraction was sequenced with three replicates. In addition, fifteen total DNA samples were submitted for sequencing, including DNA extracted from 1) the soil prior to any incubation (called initial soil), 2) the two no RDX controls (one with glucose and the other without glucose), 3) replicate microcosms amended with labeled RDX and no glucose, 4) replicate microcosms amended with unlabeled RDX and no glucose, 5) replicate

microcosms amended with labeled RDX and glucose, 6) replicate microcosms amended with unlabeled RDX and glucose, 7) replicate microcosms amended with two additions of unlabeled RDX and no glucose and 8) replicate microcosms amended with two additions of unlabeled RDX and one addition of glucose.

Illumina specific fusion primers were used to amplify the V4 region of the 16S rRNA gene and to add unique barcodes to samples in each well to enable pooling and sequencing. After the amplicons were checked on 1% agarose gel, equimolar amounts of the sample were pooled to normalize results, purified and then sequenced on the Illumina MiSeq™ Personal Sequencing System. The amplicon sequencing data in the fastq file format was analyzed on Mothur version 1.33.0 from Patrick D. Schloss Laboratory (Schloss 2009) using the MiSeq standard operating procedure (Schloss 2013). Barcode information was removed from the sequence data and contiguous sequences were created using the forward and reverse reads, were analyzed for errors and then classified. Samples were checked for the proper read length (<275 bp), ambiguous bases and homopolymer length greater than 8 to eliminate such sequences. These sequences were then aligned with the SILVA bacteria database (Pruesse et al. 2007) for the V4 region. Chimeras, mitochondrial and chloroplast lineage sequences were removed and then the sequences were classified into OTU's. The OTUs were then grouped into taxonomical levels with corresponding confidence levels. Rarefaction curves, Cho1 and Shannon values were determined for all DNA extracts using Mothur. Illumina sequencing data was deposited in the NCBI Sequence Read Archive under Bioproject: SRP049644, Biosample: SRS741275 and Bioexperiment: SRR1646680.

4.2.6. Data Analysis

The most abundant phylotypes (>1% relative abundance) in all fifteen DNA extracts were determined and compared to ascertain differences and similarities between the microbial communities. The relative abundance of phylotypes was compared between the no RDX controls (initial soil and no RDX amended microcosms) to determine which phylotypes experienced an increase in abundance following exposure to RDX. In addition, the relative abundance (%) of the most abundant phylotypes in the fractions of the labeled RDX amended samples were determined. These abundance values were then compared to values from the fractions obtained

from the unlabeled RDX amended samples. The purpose of this comparison was to determine which phylotypes had incorporated the ^{15}N or ^{13}C label from RDX and would thus be found in the heavier fractions. To determine if the differences noted were significant, two-tailed T-tests were performed 1) to compare the relative abundance of the five enriched phylotypes between the initial soils, the no RDX controls and the RDX amended samples and 2) the three enriched phylotypes in the SIP fractions from the labeled and unlabeled RDX amended samples.

4.3. Results

RDX degradation occurred in all live microcosms within 30 days, whereas no removal of RDX was noted in the abiotic controls (data not shown). In the live microcosms, additional HPLC peaks appeared and when these were compared to analytical standards, they were identified as the mono-, di- and tri-nitroso derivatives of RDX. By day 30, all additional peaks had disappeared and DNA was extracted at this time. An extra amendment of RDX was added to four microcosms and this was removed (as were the metabolites) within 20 days. Again, DNA was extracted at this time.

High throughput sequencing was conducted on fifteen total genomic DNA extracts to investigate the microbial communities present following each treatment. For this, the most abundant phylotypes (>1% relative abundance) were compared between the microbial communities of each sample (Figure 1). The most abundant phylotypes in the initial soil microbial community was clearly different from the most abundant phylotypes in the other samples. For example, GP4 was the most abundant phylotype in the initial soil (12.8%), however in the other communities, it was present only at a low level (<2.1%). In addition, the three most common phylotypes in the other samples (unclassified *Pseudomonadaceae*, *Acinetobacter* and *Gracilibacter*) were present only at low levels in the initial soil community (<0.07%). As expected, the microbial communities were similar between the microcosms amended with labeled and unlabeled RDX. The addition of glucose also did not greatly change the most abundant phylotypes present. The four most abundant phylotypes in the microcosms amended with RDX in the absence of glucose were *unclassified Pseudomonadaceae*, *Acinetobacter*, *Gracilibacter* and *unclassified Bacteroidetes*. Whereas, in the presence of glucose and RDX, the four most abundant phylotypes were *Pseudomonadaceae*, *Acinetobacter*, *Gracilibacter* and

Comamonas. Further, the communities that received two amendments of RDX were similar to those that received only one amendment.

Rarefaction curves (Figure 2) indicated that the majority of the populations were represented as these started to plateau for all of the samples. In contrast, the curve for the initial soil microbial community was still increasing, suggesting additional data is needed to represent the complete diversity of this sample. The total number of OTUs or species richness was estimated using the Chao1 estimator (Table 2). In general, the RDX and glucose amended microcosms appeared to have greater species richness compared to the RDX amended microcosms that did not receive glucose. The Chao1 value for the initial soil was notably higher than all of the other samples. The Shannon diversity value for the initial soil was also higher than all other Shannon values (Table 2). Again, the glucose amended samples illustrated higher Shannon values compared to the samples that were not amended with glucose.

To determine which microorganisms were enriched following RDX degradation, the relative abundance of each population in the RDX amended microcosms was compared to the initial soil and the no RDX control microcosms. Phylotypes with greater relative abundance in the RDX amended microcosms compared to the no RDX controls are shown (Figure 3). These differences were statistically significant (Table 3). For the glucose amended samples, five bacteria exhibited an increase over the controls (Figure 3a). From these, three exhibited a larger increase in abundance compared to the controls, including unclassified *Pseudomonadaceae*, *Pseudomonas* and *Comamonas*. For the microbial communities in the absence of glucose, *Acinetobacter* exhibited the greatest increase compared to the initial soil and no RDX controls (Figure 3b). Although, the abundance of *Acinetobacter* decreased at the second time point, perhaps as a result of competition between the other phylotypes. In the absence of glucose, *Rhodococcus* (a genus containing many known RDX degrading isolates) also increased in abundance, although it was at a much lower level than *Acinetobacter*. Interestingly, both treatments resulted in a greater relative abundance of *Comamonas* and *Sedimentibacter* in the RDX amended samples compared to the initial soil and no RDX controls. *Sedimentibacter* exhibited an increase primarily at Time 2, perhaps indicating slower growth within this community.

Total DNA samples from the labeled and unlabeled RDX amended microcosms for both the glucose and no glucose samples were subject to ultracentrifugation and fractioning. As

expected, the buoyant density of each fraction decreased as additional fractions were collected. The DNA concentration in each fraction was also determined (Figure 4). The DNA concentration was higher in the heavier fractions for the labeled RDX amended samples compared to the unlabeled RDX amended samples, indicating an enrichment of nucleic acids with ^{13}C and/or ^{15}N . This pattern occurred in both the glucose (Figure 4a) and the no glucose samples (Figure 4b). The heavy fractions were submitted for Illumina sequencing (three replicates for each) to determine which phylotypes were responsible for label uptake.

The three most abundant phylotypes in the heavy fractions from the labeled RDX amended samples were determined for both treatments (Figure 5). The relative abundances of each of the three phylotypes were statistically different between the fractions from the labeled and unlabeled RDX amended microcosms (Table 1). The sequencing data from the three replicates were similar, resulting in small error bars. From this analysis, only one phylotype (unclassified *Pseudomonadaceae*) was similar between treatments and, in both cases, it was the most dominant phylotype in the heavy fractions. In the glucose amended heavy fractions *Comamonas* and *Anaeromyxobacter* were also present, but at a much lower level compared to unclassified *Pseudomonadaceae* (Figure 5a). In the heavy fractions from the no glucose samples, *Azohydromonas* and *Rhodococcus* were present and again this occurred at a much lower level compared to the unclassified *Pseudomonadaceae* phylotype (Figure 5b). These data indicate unclassified *Pseudomonadaceae* were primarily responsible for label uptake in the soil microcosms, both in the absence and presence of glucose.

4.4. Discussion and Conclusions

The potential for any site to biodegrade RDX is particularly difficult to predict because of the large number of RDX degrading isolates and the lack of correlation between these isolates and the species found at contaminated sites. To address this, the current study combined SIP and high throughput sequencing to provide an in depth analysis of the microorganisms linked to RDX degradation in samples from a Navy Base. The study utilized two layers of information to link RDX degradation with microorganism identity. Firstly, phylotypes illustrating an increase in relative abundance following RDX degradation, compared to controls (initial soil and samples with no RDX added) were determined. This approach is similar to using quantitative PCR to document growth on a substrate for specific phylotypes within mixed communities. It cannot be

definitely stated that these microorganisms were growing using RDX as a carbon, nitrogen or energy source. However, because their abundance increased following RDX degradation, they were clearly benefiting from this process. Further, in replicate samples not exposed to RDX, this increase did not occur. Secondly, SIP was used to identify which microorganisms were involved in label uptake from RDX. Combining the results from these two approaches has provided an enhanced data set for documenting the microorganisms involved in RDX degradation.

To date, a range of molecular methods have been used to examine microbial communities at RDX contaminated sites (Fuller et al. 2010; Kwon and Finneran 2010; Kwon et al. 2011; Moshe et al. 2009; Ringelberg et al. 2008; Ronen et al. 2008). Only recently (2013), has high throughput sequencing (pyrosequencing, not in combination with SIP) been applied to examine RDX degrading microbial communities (Livermore et al. 2013). Four studies have applied SIP to investigate RDX degradation (Andeer et al. 2013; Cho et al. 2013; Jayamani et al. 2013; Roh et al. 2009). SIP has the advantage in that the labeled carbon or nitrogen derived from RDX can be linked to microorganism identity, providing more robust information on the microorganisms involved in RDX degradation. The current study is the first to combine SIP with high throughput sequencing to investigate the microorganisms responsible for RDX degradation. To our knowledge, SIP has been used in combination with traditional Sanger sequencing in four studies to examine RDX degradation (Andeer et al. 2013; Cho et al. 2013; Jayamani et al. 2013; Roh et al. 2009). One study revealed the importance of *Sphingobacteriales* in label uptake (Jayamani et al. 2013). Another SIP study identified five phylotypes similar to known RDX degraders and ten phylotypes not previously linked to RDX degradation (Roh et al. 2009). The authors reported that several of these phylotypes classified within the genus *Pseudomonas*. The authors did not report any enrichment of phylotypes from the *Betaproteobacteria*, indicating they did not find enrichment of the genus *Comamonas*. A more recent SIP study also reported the importance of *Pseudomonas* phylotypes in label uptake (Cho et al. 2013). However, the research also reported label uptake over a range of phyla, including *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Cho et al. 2013). The fourth SIP study found that *Rhodococcus* exhibited the greatest amount of label uptake (Andeer et al. 2013). From the other seven phylotypes that were associated with label uptake, one classified (*Variovorax*) within the same family as *Comamonas* and another classified within the same order (*Burkholderiales*). The results obtained in the current study agree with previous SIP studies, indicating the importance of

phylotypes within the families *Pseudomonadaceae* and *Comamonadaceae* in label uptake from RDX. In addition, similar to previous studies, the current research also found that *Rhodococcus* was associated with label uptake. Taken together, these data indicate that primers designed towards these phylotypes would be advantageous for investigating the feasibility of bioremediation across RDX contaminated sites.

Known RDX degrading isolates classify within four phyla, the *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*. The phylum *Firmicutes* contains many RDX degrading bacteria (Adrian and Arnett 2004; Bhushan et al. 2004; Kwon and Finneran 2008b; Regan and Crawford 1994; Sherburne et al. 2005; Singh et al. 2009; Zhang and Hughes 2003; Zhao et al. 2003). In the current study, unclassified *Clostridiaceae* 1 and *Sedimentibacter* were enriched following RDX degradation, but were not linked with label uptake from RDX. The phylum *Actinobacteria* also contains RDX degrading isolates including many within the genus *Rhodococcus* (Bernstein et al. 2011; Bhushan et al. 2003; Coleman et al. 1998; Coleman et al. 2002; Fournier et al. 2002; Nejidat et al. 2008; Seth-Smith et al. 2008; Seth-Smith et al. 2002). Other *Actinobacteria* isolates include *Williamsia* sp. KTR4 (Thompson et al. 2005), *Gordonia* sp. KTR9 (Indest et al. 2010; Thompson et al. 2005) and *Gordonia* sp. YY1 (Ronen et al. 2008). The data from the current study indicate *Rhodococcus* phylotypes increased in abundance following RDX degradation and the SIP data suggest these phylotypes are responsible for lower levels of label uptake.

RDX degrading isolates in the phylum *Proteobacteria* are found within the *Alpha*-, *Gamma*- and *Deltaproteobacteria* (Cupples 2013). In the current study, *Anaeromyxobacter* was the only *Deltaproteobacteria* implicated in label uptake and previous research has indicated this genus is capable of RDX degradation (Kwon and Finneran 2008b). In the *Betaproteobacteria*, two phylotypes were associated with label uptake (*Comamonas* was particularly enriched and *Azohydromonas* was enriched to a lower level). Interestingly, no pure cultures of *Betaproteobacteria* have been shown to be capable of RDX degradation. The most dominant phylotype responsible for label uptake was a *Gammaproteobacteria*, unclassified *Pseudomonadaceae*. These results collaborate previous research, as many *Gammaproteobacteria* isolates are able to degrade RDX (Kitts et al. 2000; Pudge et al. 2003; Young et al. 1997; Zhao et al. 2002). When the unclassified *Pseudomonadaceae* partial 16S rRNA gene sequence was compared to those in Genbank it was found to be 100% similar to

twenty uncultured bacterium sequences, seven *Pseudomonas* sequences, and four *Azotobacter tropicalis* sequences. In the current study *Pseudomonas* was also enriched following RDX degradation and again *Pseudomonas* isolates have been shown to be able to metabolize RDX (Cho et al. 2008; Singh et al. 2009).

In summary, SIP was combined with high throughput sequencing to investigate the microorganisms involved in RDX degradation. The two phylotypes associated with label uptake included *Comamonas* and an unclassified *Pseudomonadaceae*. The high throughput sequencing data indicated these two phylotypes also increased in abundance following RDX degradation compared to the controls. These data suggest the presence of these microorganisms at contaminated sites should enhance RDX remediation efforts.

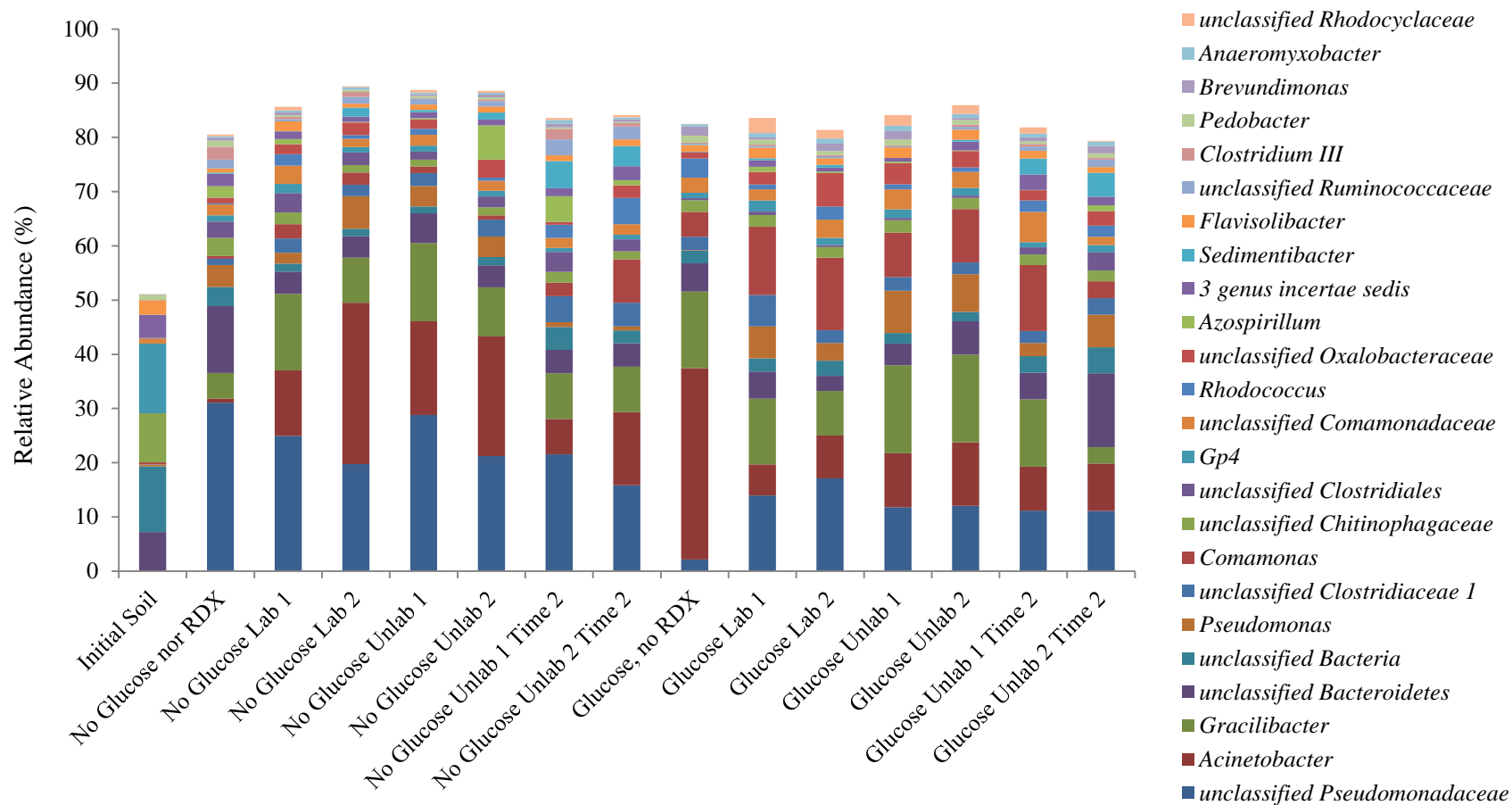


Figure 4.1. Relative abundance of the most common phylotypes in RDX amended microcosms (with and without glucose) compared to their abundance in the initial soil community and no RDX microcosms.

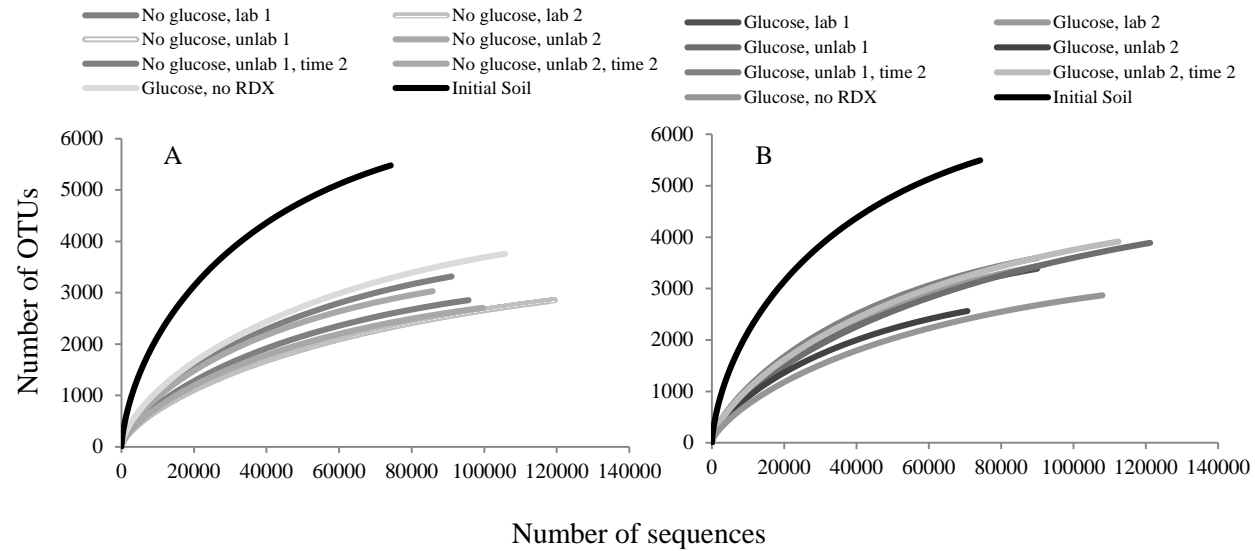


Figure 4.2. Rarefaction curves for the total microbial communities in the initial soil, RDX amended microcosms and the no RDX controls for both the no glucose amended (A) and glucose amended (B) microcosms.

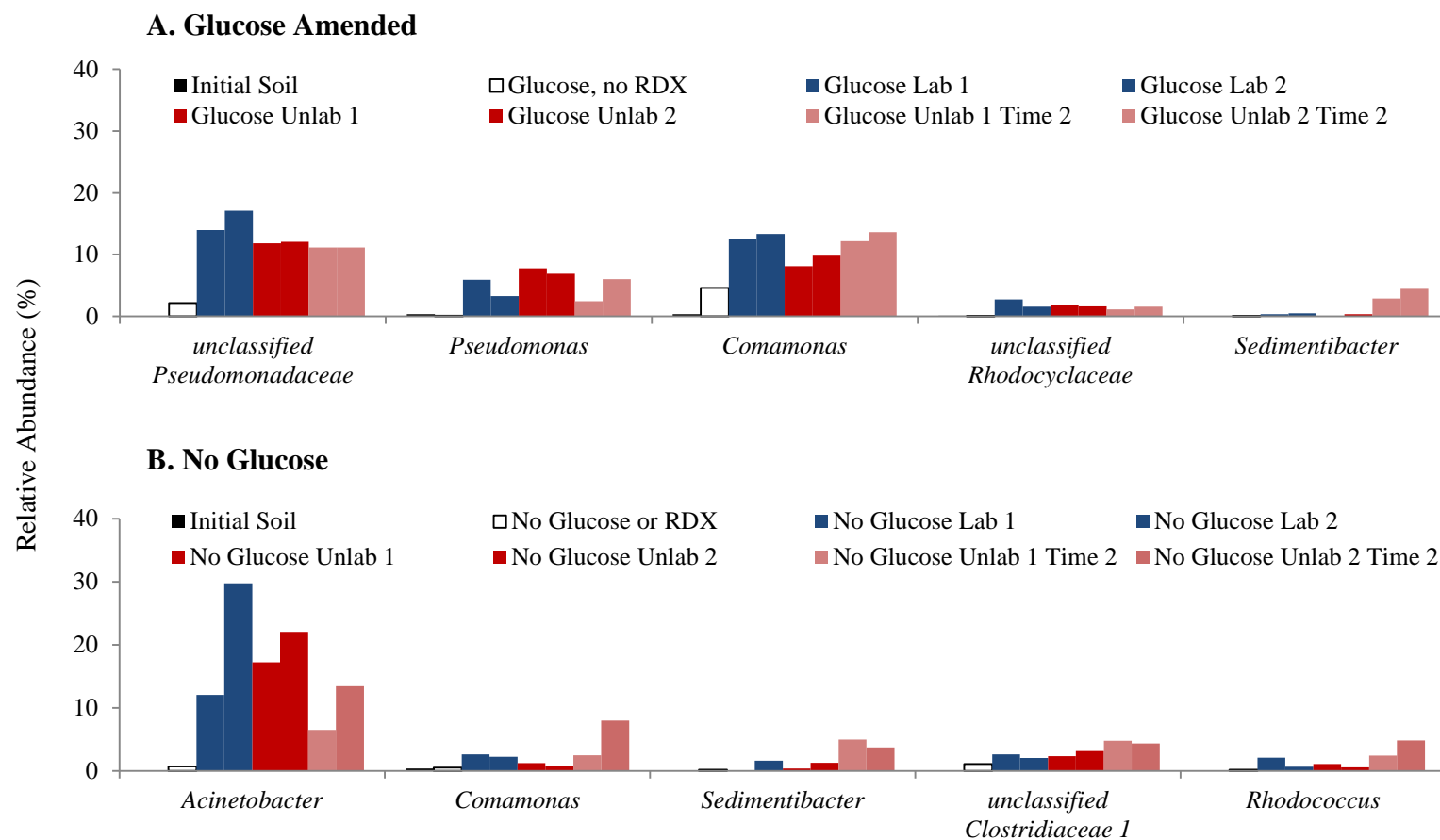


Figure 4.3. Relative abundance of phylotypes illustrating a difference in relative abundance between the RDX amended microcosms and the no RDX controls (initial soil and no RDX microcosm) in the glucose amended microcosms (A) and in the microcosms that were not amended with glucose (B).

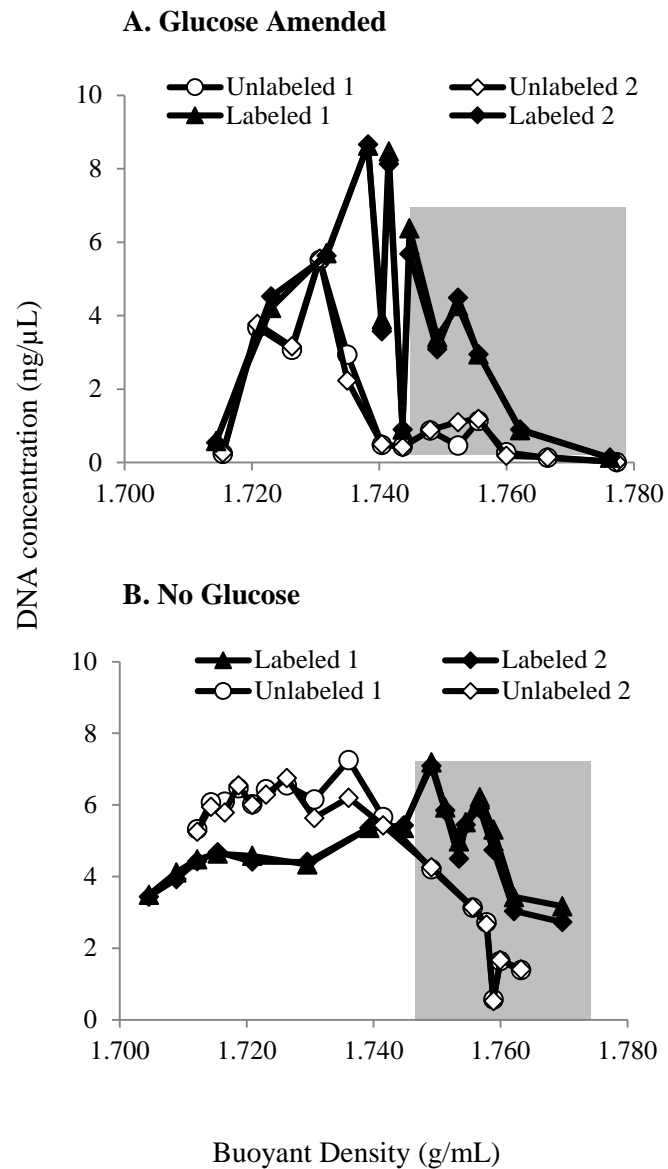
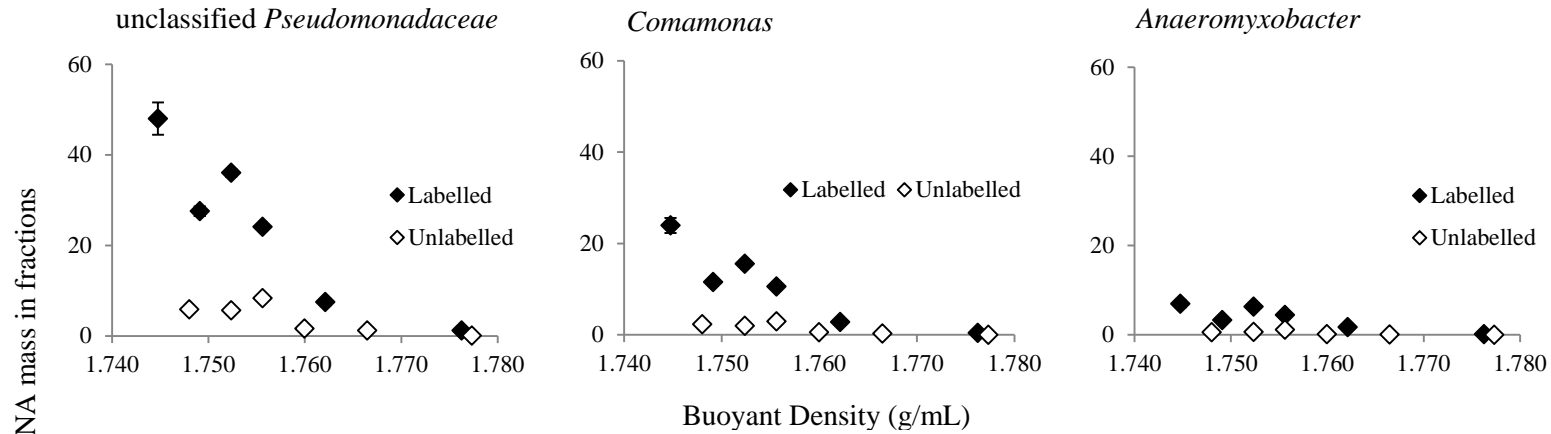


Figure 4.4. DNA concentration (ng/μL) in fractions across buoyant density gradients obtained from samples amended with labeled and unlabeled RDX, with (A) or without (B) the addition of glucose. Replicate lines represent duplicate DNA measurements. The shaded area represents the fractions analyzed with Illumina sequencing.

A. Glucose Amended



B. No Glucose

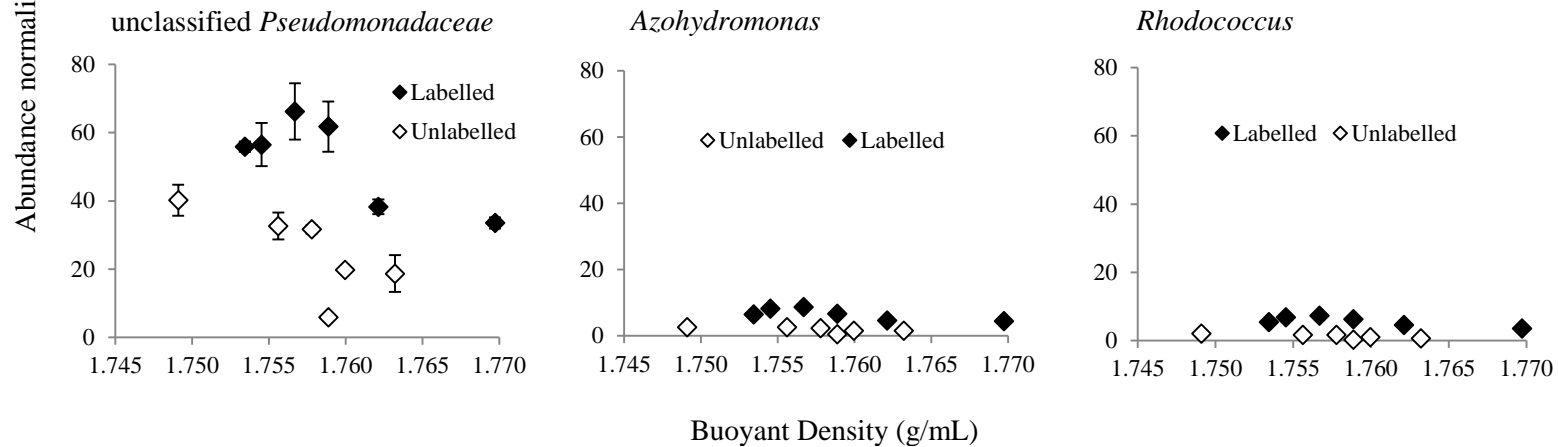


Figure 4.5. The three most abundant phylotypes in the heavy fractions from the labeled RDX amended microcosms compared to their abundance in fractions of similar buoyant density from the unlabeled RDX amended microcosms. Data are shown from those amended with glucose (A, top three graphs) and those not amended with glucose (B, bottom three graphs). The y-axis represents relative abundance in each fraction normalized by the DNA mass in each fraction (relative abundance times the DNA mass (ng)). Error bars represent standard deviations from three replicates and may be too small to be seen.

Table 4.1. Summary of sample names, amendments and time for DNA extraction.

Sample name	RDX	Glucose	DNA extraction
Initial soil	No addition	No addition	Day 0
No glucose with RDX 1	unlabeled RDX	No addition	No extraction, abiotic control
No glucose with RDX 2	unlabeled RDX	No addition	No extraction, abiotic control
No glucose nor RDX	No addition	No addition	Day 30
No glucose Lab 1	¹³ C, ¹⁵ N-RDX	No addition	Day 30
No glucose Lab 2	¹³ C, ¹⁵ N-RDX	No addition	Day 30
No glucose Unlab 1	unlabeled RDX	No addition	Day 30
No glucose Unlab 2	unlabeled RDX	No addition	Day 30
No glucose Unlab 1 Time 2	unlabeled RDX	No addition	Day 50
No glucose Unlab 2 Time 2	unlabeled RDX	No addition	Day 50
Glucose with RDX 1	unlabeled RDX	Addition	No extraction, abiotic control
Glucose with RDX 2	unlabeled RDX	Addition	No extraction, abiotic control
Glucose, no RDX	No addition	Addition	Day 30
Glucose Lab 1	¹³ C, ¹⁵ N-RDX	Addition	Day 30
Glucose Lab 2	¹³ C, ¹⁵ N-RDX	Addition	Day 30
Glucose Unlab 1	unlabeled RDX	Addition	Day 30
Glucose Unlab 2	unlabeled RDX	Addition	Day 30
Glucose Unlab 1 Time 2	unlabeled RDX	Addition	Day 50
Glucose Unlab 2 Time 2	unlabeled RDX	Addition	Day 50

	Chao1	Lower 95% CI	Upper 95% CI	Shannon	Lower 95% CI	Upper 95% CI
Initial Soil	6444	6331	6574	6.6	6.5	6.6
Glucose, no RDX	3469	3379	3575	3.4	3.4	3.4
Glucose, lab 1	4019	3928	4125	4.3	4.2	4.3
Glucose, lab 2	4404	4277	4552	4.3	4.3	4.3
Glucose, unlab 1	4974	4839	5130	4.2	4.2	4.2
Glucose, unlab 2	3183	3086	3296	4.1	4.0	4.1
Glucose, unlab 1, time 2	4261	4168	4368	4.4	4.4	4.4
Glucose, unlab 2, time 2	4718	4612	4840	4.5	4.5	4.5
No glucose, no RDX	4531	4427	4653	4.3	4.3	4.3
No glucose, lab 1	3435	3347	3538	3.8	3.7	3.8
No glucose, lab 2	3502	3406	3614	3.3	3.3	3.3
No glucose, unlab 1	3628	3512	3764	3.3	3.3	3.3
No glucose, unlab 2	3238	3155	3337	3.6	3.6	3.6
No glucose, unlab 1, time 2	4029	3929	4145	4.3	4.3	4.3
No glucose, unlab 2, time 2	3725	3622	3845	4.1	4.1	4.2

Table 4.2. Chao1 and Shannon and lower and upper confidence intervals (CI) values for all microbial communities.

Table 4.3. T-test (two-tailed) results from a comparison of the relative abundance of five phylotypes with and without RDX. Also, results are shown from comparing the relative abundance of the three enriched phylotypes in fractions from the labeled and unlabeled RDX amended microcosms (SIP results).

Samples	Calculated t value	Critical t value (two-tailed)	p value
Five phylotypes enriched phylotypes (with glucose)			
Initial soil compared to with RDX (all five phylotypes)	-6.74	2.05	2.12×10^{-7}
Without RDX compared to with RDX	-3.99	2.13	1.17×10^{-3}
Five phylotypes enriched phylotypes (without glucose)			
Initial soil compared to with RDX	-4.18	2.05	2.40×10^{-4}
Without RDX compared to with RDX	-3.78	2.04	7.00×10^{-4}
Fractions: labeled compared to unlabeled RDX (with glucose)			
Unclassified <i>Pseudomonadaceae</i>	5.14	2.10	6.93×10^{-5}
<i>Comamonas</i>	4.91	2.10	1.12×10^{-4}
<i>Anaeromyxobacter</i>	5.65	2.10	2.31×10^{-5}
Fractions: labeled compared to unlabeled RDX (without glucose)			
Unclassified <i>Pseudomonadaceae</i>	6.50	2.03	1.91×10^{-7}
<i>Azohydromonas</i>	10.70	2.06	1.30×10^{-10}
<i>Rhodococcus</i>	12.04	2.06	1.16×10^{-11}

5.0. Microbial Community Characterization and Functional Gene Quantification in RDX Degrading Microcosms Derived from Sediment and Groundwater at Two Naval Sites

This chapter was published (Paes Wilson and Cupples In press) as follows:

Paes Wilson F, Cupples AM (in press) Microbial community characterization and functional gene quantification in RDX degrading microcosms derived from sediment and groundwater at two Naval sites. Applied Microbiology and Biotechnology.

Abstract

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has long been recognized as a problematic environmental pollutant and efforts to remediate contaminated soils, sediments and groundwater have been ongoing for decades. In recent years, much interest has focused on using bioremediation to clean up these sites. The current study investigated the microorganisms (16S rRNA genes, Illumina) and functional genes (*xenA*, *xenB* and *xplA*) linked to RDX biodegradation in microcosms composed of sediment or groundwater from two Navy sites. For this, experiments included sediment samples from three depths (5 to 30 feet) from two wells located in one Navy site. In addition, the groundwater upstream and downstream of an emulsified oil biobarrier was examined from another Navy site. Further, for the groundwater

experiments, the effect of glucose addition was explored. For the sediment experiments, the most enriched phylotypes during RDX degradation varied over time, by depth and well locations. However, several trends were noted, including the enrichment of *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and *Sporolactobacillus* in the sediment microcosms. For the groundwater based experiments, *Pseudomonas*, unclassified *Rhodocyclaceae*, *Sphingomonas* and *Rhodococcus* were also highly abundant during RDX degradation. The abundance of both *xplA* and *xenA* significantly increased during RDX degradation compared to the control microcosms for many treatments (both groundwater and sediment microcosms). In a limited number of microcosms, the copy number of the *xenB* gene increased. Phylotype data were correlated with functional gene data to highlight potentially important biomarkers for RDX biodegradation at these two Navy sites.

5.1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is the most widely used military explosive (Rylott et al. 2011a). The continued global demand for munitions suggests that RDX manufacture and use will occur for the foreseeable future. RDX has long been recognized as a problematic pollutant, with contamination being particularly problematic on military training ranges where energetic materials are detonated on a regular basis. Pollution can originate from unexploded ordinance or from nonpoint sources. In either case, RDX tends to be recalcitrant and can remain in the soil and also move to groundwater. Groundwater contamination is a cause for concern because RDX is classified as a possible human carcinogen by the EPA. In recent years, much interest has focused on using bioremediation to clean up these sites; however the microorganisms responsible for *in situ* degradation are generally unknown .

To determine which microorganisms are linked to RDX degradation at contaminated sites, methods such as stable isotope probing (SIP) and high throughput sequencing have been utilized (Andeer et al. 2013; Cho et al. 2015; Cho et al. 2013; Jayamani and Cupples 2015a; Jayamani and Cupples 2015b). For example, 5 phylotypes similar to known RDX degraders and 10 phylotypes not previously linked to RDX degradation were associated with RDX degradation in samples from Picatinny Arsenal (NJ) . Another set of SIP experiments showed the importance of *Pseudomonas* phylotypes in label uptake (Cho et al. 2013). In contrast, *Rhodococcus* was

identified by SIP as the primary degrader in soils from the Eglin Air Force Base bombing range (Eglin, FL) (Andeer et al. 2013). Recently, also through the use of SIP, *Pseudomonadaceae* and *Comamonas* were identified as RDX-degraders in soil (Jayamani and Cupples 2015b). The growing literature on phylotypes identified as RDX degraders at contaminated sites has the potential to improve current bioremediation approaches. However, to date, limited information exists on the distribution of RDX degraders at different depths at such sites. This information is important because of the risk of RDX movement from soils to groundwater.

Bioremediation efforts have been significantly enhanced in the past decade using functional gene data. For example, the dehalogenase reductive genes responsible for vinyl chloride degradation, *bvcA* and *vcrA*, are routinely quantified in groundwater from chlorinated-solvent contaminated sites to determine the dechlorination potential of *in situ* dechlorinators (Hatt et al. 2013; Kanitkar et al. 2016; Stedtfeld et al. 2014). A number of functional genes have been associated with RDX biodegradation, including two that encode for flavin mononucleotide-containing oxidoreductases (called xenobiotic reductases) from *Pseudomonas putida* II-B (*xenA*) and *Pseudomonas fluorescens* I-C (*xenB*) (Fuller et al. 2009). For these, the primary RDX degradation path is through methylenedinitramine (MEDINA) and then to formaldehyde. Both genes have been cloned, sequenced and characterized (Blehert et al. 1999), providing an excellent opportunity to examine gene abundance at contaminated sites. The enzyme diaphorase from the anaerobic bacterium *Clostridium kluyveri* has also been linked to RDX degradation (Bhushan et al. 2002) and the gene sequence (*diaA*) encoding diaphorase from *C. kluyveri* has been determined (Chakraborty et al. 2008). Another functional gene related to RDX degradation is *hydA* from *C. acetobutylicum* (Watrous et al. 2003). Also, an oxygen-insensitive (type I) NADPH nitroreductases (*nsfT*) enzyme able to degrade RDX has been detected in enteric bacteria *Enterobacter cloacae* and *Morganella morganii*.

Among all of the functional genes associated with RDX degradation, *xplA* has perhaps been the most studied. It has been identified in isolates of the genera *Rhodococcus*, *Gordonia*, *Williamsia* (all three are in the suborder *Corynebacterineae*, phylum *Actinobacteria*) and *Microbacterium* (suborder *Micrococcineae*, phylum *Actinobacteria*) (Andeer et al. 2009; Bernstein et al. 2011; Coleman et al. 1998; Indest et al. 2007; Nejdat et al. 2008; Rylott et al. 2011a). The RDX breakdown products, 4-nitro-2,4-diazabutanal (NDAB) and MEDINA have been noted for *Rhodococcus* spp. during RDX degradation. Both *xplA* and its partnering *xplB*

gene (encodes a flavin reductase) are carried by a plasmid, and research suggests that they are likely part of a class I transposable element (Andeer et al. 2009). *XplA* has been associated with RDX degradation under aerobic and anaerobic conditions (Halasz et al. 2012; Jackson et al. 2007).

A limited number of studies have investigated the occurrence of the genes associated with RDX biodegradation at contaminated sites. One project examined RDX-contaminated groundwater from two sites (Pueblo Chemical Depot and Picatinny Arsenal) where bioremediation was ongoing. Using conventional PCR, the authors were not able to detect any of the five RDX functional genes investigated (*xplA*, *hydA*, *onr*, *xenA*, *xenB*) (Fuller et al. 2010). SIP studies using samples from contaminated sites have detected *xplA* in heavy SIP fractions (label enriched) and in isolates from these sites (Andeer et al. 2013). A recent metagenomic study, which focused on ovine rumen microbiota capable of RDX degradation found sequence homologues to five RDX-degrading genes (*diaA*, *xenA*, *xenB*, *xplA* and *xplB*) (Li et al. 2014). Among these, *diaA* was the most abundant, followed by *xenA* and *xenB*. In contrast, *xplA* and *xplB* were barely detectable and homologues of *nsfI* were not detected (Chong et al. 2014). Although this research provides novel data on ovine rumen microbiota, it does not shed light on the importance of these genes at contaminated sites.

In the current study, the abundance and distribution of three functional genes (*xplA*, *xenA* and *xenB*) associated with RDX degradation was investigated using samples derived from two Naval sites. It was deemed important to include *xplA*, given the large and growing interest in this gene. The genes *xenA* and *xenB* were included because previous research indicated *Pseudomonadaceae* were important for RDX degradation in environmental samples or *in situ* (Jayamani and Cupples 2015b). In addition, the microbial community enriched during RDX biodegradation was examined using high throughput sequencing (Illumina MiSEQ).

Functional genes and community analyses were performed on sediment obtained from different depths at two wells from one Navy site. The aim was to ascertain if RDX biodegradation potential changed with depth, perhaps depending on oxygen availability or changes in microbial communities with depth. Additional experiments involved groundwater obtained from both upstream and downstream of a buffered emulsified oil biobarrier from a second Navy site. The biobarrier had been installed to facilitate bioremediation of RDX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX, another explosive) and perchlorate. To

our knowledge, this is the first study to examine these variables (functional or 16S rRNA genes) with sediment depth or surrounding a biobarrier. The aim of this research was to determine which functional genes and phylotypes are most closely linked to RDX degradation in samples from contaminated sites, so that future bioremediation endeavors can focus on the detection of these key biomarker genes.

5.2. Methods

5.2.1. Chemicals

RDX dissolved in acetonitrile was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). HPLC grade acetonitrile ($\geq 99.8\%$ purity) was purchased from EMD Chemicals Inc. (New Jersey, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher BioReagent (New Jersey, USA), or Invitrogen (Carlsbad, CA, USA), unless otherwise stated.

5.2.2. Experimental design

Sediment samples from different depths were collected from two wells (well 61 and well 58), both located on an RDX contaminated Navy base (Naval Base Kitsap, WA, hereafter, called site 1). For well 58, samples from five depths were collected (5, 10, 20, 25 and 30 feet deep) and for well 61, samples from three depths were collected (5, 10 and 20 feet deep). The sediments were collected while drilling deep borings. Groundwater samples from a second Navy site (US Department of Defense explosives testing range in Virginia, site 2) were collected from both upstream (well 10) and downstream (well 1) of an installed buffered emulsified oil biobarrier. The biobarrier (100 ft) was placed perpendicular to the RDX plume by injecting a mixture of EOS 550LS (4% v:v) plus CoBupH (0.75% v:v) (EOS Remediation, <http://www.eosremediation.com>) at 20 injection wells in March, 2013. A second injection consisting of 9.5 % EOS 550LS and 0.75% EOS CoBupH in the central part of the barrier was conducted approximately 20 months later (October, 2014). Samples for this study were collected 5 months after the second injection (March 2015). The upgradient well was 10 ft from the biobarrier and the downgradient well was 2.5 ft from the biobarrier. Groundwater chemistry was as follows: pH of 6.31, DO of 0.21 mg/L, ORP of -129.3 mV, TOC of 62.0 mg/L, SO_4^{2-} of 2.6 mg/L, NO_3^- as N of <0.2 mg/L, NO_2^- as N of <0.2 mg/L and Cl^- of 3.4 mg/L for the downgradient well and pH of 4.61, DO of 2.89 mg/L, ORP of 296.0 mV, TOC of 1.53 mg/L,

SO_4^{2-} of 11.2 mg/L, NO_3^- as N of 3.6 mg/L, NO_2^- as N of <0.2 mg/L and Cl^- of 3.0 mg/L for the upgradient well. At the time of sample collection, the RDX in the upgradient well was 116 ug/L while that in downgradient well was below detection (< 0.03 ug/L). The following acids were present in the groundwater well downstream from the biobarrier: acetic acid (107 mg/L), propionic acid (57.4 mg/L), butyric acid (6.2 mg/L) and pyruvic acid (6.4 mg/L). These acids were below 2 mg/L in the groundwater well upstream of the biobarrier. The biobarrier proved to be very effective for promoting RDX, HMX and perchlorate biodegradation in the shallow aquifer (Paul Hatzinger, CB&I, personal communication). All samples were shipped on ice and were refrigerated until use.

From each sample, triplicates of live and killed abiotic controls (autoclaved) microcosms were prepared. The microcosms were established as previously described, with modifications. Briefly, each microcosm consisted of a 60 mL serum bottle containing 4 mL of a minimal salts media (28, 34), 10 mg L⁻¹ RDX dissolved in acetonitrile (nitrogen sources), and either 2 g sediment (wet weight) or 1 mL groundwater. Live microcosms with an equal volume of acetonitrile, but not containing RDX, were also prepared as described above, in triplicate, for all sediment and groundwater samples (called No RDX control microcosms). Finally, because an additional carbon source is commonly used to accelerate RDX biodegradation, live and killed abiotic control microcosms were prepared for the groundwater samples with the addition of 200 mg L⁻¹ glucose. All microcosms were sealed with rubber stoppers and aluminum seals and were covered with aluminum foil to prevent photodegradation. The sediment microcosms were incubated between 6 to 19 weeks, while the groundwater microcosms were incubated between 9 to 15 weeks. All microcosms were stored at room temperature (~20 °C) without shaking. The microcosms remained closed for the duration of the experiments. Although oxygen concentrations were not measured, based on previous research in our laboratory, it was likely that oxygen was depleted before RDX degradation occurred. To ensure no oxygen entered the microcosms, samples were removed using a needle and syringe through the rubber septum.

5.2.3. Analytical methods

RDX concentrations were determined using high performance liquid chromatography (HPLC) as previously described (Thompson et al. 2005) with modifications. For each measurement, a 0.2 mL aliquot was removed with a 1 mL BD syringe (21-gauge needle) and

combined with an equal volume of acetonitrile in a 1.7 mL sterile microcentrifuge tube. The tubes were shaken for 30 minutes at room temperature to extract RDX. The samples were then centrifuged for 5 minutes (10,000 x g) and the supernatant was filtered using acetonitrile-wetted filters (PVDF, 0.22 µm, Whatman) into HPLC amber vials (Sigma-Aldrich, St. Louis, MO, USA). External standards for the calibration curve were prepared with a dilution factor of 2 to account for the sample dilution at the liquid-liquid extraction step. The HPLC parameters were as follows: column injector volume, 20 µL for samples and standards; isocratic conditions (40% acetonitrile and 60% 0.1% H₃PO₄⁻ acidified water, 1 mL min⁻¹); Supelco C18 (25 cm X 4.6 mm, 5 µm); Perkin Elmers Series 200 autosampler; PE binary LC Pump 250; Waters UV detector; wavelength 255 nm (the detection limit was 500 µg L⁻¹).

5.2.4. DNA extraction

DNA was extracted from live microcosms inoculated with sediment or groundwater (RDX amended microcosms and No RDX control microcosms) from an aliquot of 0.5 mL (removed using 1 mL BD syringe, 21-gauge needle). For the sediment microcosms (site 1), DNA extraction occurred for aliquots removed at two time points (well 61 at days 45 and 90; well 58 at days 90 and 130). For each well, microcosms constructed from sediment from three depths were investigated (5, 10 and 25 feet for well 58 and 5, 10 and 20 feet for well 61). For the groundwater microcosms (site 2), DNA was extracted at days 67 and 100 for the downstream and upstream groundwater samples, respectively. Also, DNA was extracted from the groundwater microcosms both amended and not amended with glucose. In addition, DNA was extracted in triplicate from all original sediment and water samples collected at the sites to establish the baseline microbial community (called initial sediment or initial groundwater). The Power Sediment DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) was used to all DNA extractions following the manufacturer's recommended procedure.

5.2.5. Quantitative PCR (qPCR)

The three functional genes associated with RDX degradation were quantified using qPCR. Quantification was performed in triplicate using DNA extracted from the RDX degrading microcosms, the No RDX control microcosms and the initial samples (groundwater or sediment). Primers were designed to target *xplA*, *xenA* and *xenB* using Primer-BLAST

(<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1) and the primers were manufactured by Integrated DNA Technologies (IDT, Coralville, IA). Although primers have previously been developed for several of these genes, new primers were designed in this study to be current with the new sequence data available in GenBank.

Amplification and qPCR measurements were conducted in a Chromo 4 Real-Time PCR Cycler (Bio-Rad, Philadelphia, PA) using a QuantiTect SYBR Green PCR Kit (Qiagen Inc.) and the primer sets for *xplA*, *xenA* and *xenB*. Each 25 μ L qPCR reaction contained 12.5 μ L QuantiTect SYBR Green PCR Master Mix solution, 1.25 μ L each 10 μ M primer, 8 μ L DNA-free water and 2 μ L DNA template. The thermal protocol consisted of an initial denaturation (95°C, 15 min), 40 cycles (95°C, 15 s; 58°C, 20 s; 72°C 20 s), and a terminal extension step (72°C, 2 min). Melting curves were constructed from 55°C to 95°C and read every 0.6°C for 2s.

Standard curves were developed in triplicate using plasmid DNA containing partial *xplA*, *xenA* or *xenB* sequences (GenScript, Piscataway, NJ) (Table 1). Gene copy numbers were calculated as described previously (Ritalahti 2006) (2710 bp plasmid size, in addition to inserts of 382 bp for *xplA*, 324 bp for *xenA* and 351 bp for *xenB*). The standard curve ranges were 10^3 - 10^9 or 10^3 - 10^{10} and the overall qPCR efficiency was 100.2% (Table 2).

5.2.6. MiSEQ Illumina sequencing and data analysis

Total genomic DNA extracted from live microcosms (sediment and groundwater microcosms) and the initial sediment and groundwater samples was submitted for high throughput sequencing (MiSEQ Illumina Sequencing) at the Research Technology Support Facility (RTSF) at Michigan State University (MSU, East Lansing, MI).

PCR and Illumina sequencing were performed at RTSF using a previously described protocol (Caporaso et al. 2011), which involves the amplification of the V4 region of the 16S rRNA gene using a set of multiplex indexed primers. Following amplification, individual reactions were quantified (Picogreen assay), a pool of equimolar amounts of each was made, and these were purified using Ampure XP beads. A final gel purification step was included to ensure non-specific products were eliminated. The combined library was loaded onto the Illumina MiSEQ Platform using a standard MiSEQ paired end (2x250 bp) flow cell and reagent cartridge. The amplicon sequencing data generated by the Illumina MiSeq Platform (*.fastq files) were analyzed using the Mothur software following the MiSEQ standard operating procedure

developed by Schloss (http://www.mothur.org/wiki/MiSeq_SOP). This involved the construction of contigs, error and chimera removal followed by sequence alignment for OTU assignment based on the SILVA database . A summary of the MiSEQ data is provided (Table 3).

To identify which phylotypes were linked to RDX degradation, the relative abundance (%) of each was calculated and the ten most abundant phylotypes during RDX degradation for each treatment were selected. The relative abundances of these phylotypes were then compared between the RDX amended samples, the No RDX control microcosms and the initial samples. Illumina sequencing data were deposited in the NCBI Sequence Read Archive under Bioproject Number PRJ302752.

5.3. Results

5.3.1. RDX degradation

RDX degradation occurred in both sediment microcosms (wells 58 and 61) (Figures 1 and 2) and groundwater microcosms (Figure 3) amended with RDX but not in the abiotic controls, confirming biological removal. RDX degradation appeared to occur faster in microcosms derived from well 61 (Figure 2) compared to well 58 (Figure 1). For both experiments with sediment samples, no clear trends between sediment depth and RDX removal rates were observed. In general, RDX removal was greater at day 67 in the microcosms inoculated with groundwater from downstream of the biobarrier (Figure 3A, C) compared to those derived from groundwater upstream of the biobarrier (Figure 3B, D). These data suggest the biobarrier was successful for enriching an *in situ* community of RDX degrading microorganisms. Total genomic DNA extracts from all of the original samples (initial sediment and initial groundwater samples) and from the live microcosms inoculated with sediment or groundwater (RDX amended and No RDX control microcosms) were subject to qPCR to enumerate RDX functional genes and Illumina sequencing to identify the dominant phylotypes.

5.3.2. Functional gene analysis in the sediment microcosms (well 58 and 61, site 1)

In well 58 microcosms, *xplA* gene copies increased during RDX degradation (Figure 4A). Specifically, by day 130, the *xplA* gene copies were higher among the RDX amended microcosms compared to the initial samples or the no RDX controls for all three depths. For well 61 microcosms, *xplA* gene copies also increased in microcosms during RDX degradation (Figure

4D). In this case, the increase compared to the initial sample was noted at all three depths (5, 10 and 20 feet) at both time points (day 45 and day 90). Also, there was a difference in *xplA* copies between the RDX amended microcosms and the No RDX control microcosms at day 45 for all depths (Figure 4D). Control microcosms were not examined at day 90, thus no comparison could be made.

In well 58 microcosms, the number of gene copies of *xenA* was higher in the RDX amended samples compared to the initial samples or the no RDX controls at day 130 at all three depths (Figure 4B). For well 61, only the microcosms from the 10 feet depth sediments illustrated an increase in *xenA* gene copies in the RDX amended samples compared to the No RDX controls and the initial samples (Figure 4E).

In well 58 microcosms, the number of gene copies of *xenB* was higher in the RDX amended samples compared to the RDX controls at day 130 for depths 5 and 25 feet (Figure 4C). For well 61 microcosms, only the microcosms from 20 feet deep sediments illustrated an increase in *xenB* gene copies compared to the initial samples and the No RDX controls (Figure 4F). Increases in *xenB* gene copies in the RDX amended samples compared to the initial samples were observed at both time points (day 45 and day 90).

5.3.3. Functional gene analysis in the groundwater microcosms (site 2)

The three functional genes were also quantified in the initial groundwater and in microcosms containing groundwater from wells downstream (well 1) and upstream (well 10) of the biobarrier (Figure 5). As stated before, some of these microcosms were also amended with glucose. The majority of the differences noted between the RDX amended microcosms and the No RDX controls or the initial samples were observed for downstream (well 1) samples (Figure 5A, B, C). Specifically, for *xplA*, an increase was noted between the RDX amended microcosms and the initial groundwater or the No RDX control in the absence of glucose (Figure 5A).

For *xenA*, in the downstream samples, a difference was observed between the initial groundwater and the RDX amended samples (with or without glucose) as well as between the RDX amended sample and the No RDX controls (with or without glucose) (Figure 5B). In the downstream samples, for *xenB*, an increase was observed in the RDX amended samples (no glucose) compared to the initial groundwater or the No RDX controls (no glucose). Also, an increase was noted between the RDX amended samples with glucose and the initial groundwater

(Figure 5C).

The functional gene numbers from the experiments involving groundwater from well 10 (upstream of the biobarrier, Figure 5D, E, F) exhibited different trends to well 1 (Figure 5A, B, C). There was no notable difference between gene numbers for *xplA* or *xenA* for any treatment investigated (Figure 5D, E). However, *xenB* copy numbers were higher in the RDX (and glucose) amended samples compared to the No RDX controls or initial samples (Figure 5F). The samples with glucose (both with and without RDX) did not contain any detectable *xenB* genes.

5.3.4. Phylotypes enriched during RDX degradation in sediment microcosms (wells 58, 61, site 1)

For well 58, the most abundant phylotypes in the RDX amended microcosms varied between the microcosms constructed from different depths (Figure 6). Differences in enrichment patterns were also noted between the two time points (days 90 and 130). At day 90, only *Pseudomonas*, *Rhodococcus* and *Arthrobacter* were common across the abundant phylotypes at the three depths (Figure 6A, C, E). At day 130, no common phylotypes were noted among the three depths from this subgroup of abundant phylotypes (Figure 6B, D, F).

For well 58, at the most shallow depth (5 feet), *Yesinia*, *Burkholderia*, *Dyella* and *Pseudomonas* were the most dominant phylotypes at both time points in the RDX amended samples. Both *Yersinia* and *Dyella* were minor phylotypes initially and when RDX was absent (Figure 6A, B). At 10 feet, only *Rhodococcus*, *Arthrobacter* and *Brevundimonas* were dominant at both time points (Figure 6C, D). At 10 feet, *Pseudomonas* and *Acidovorax* were the most abundant in the RDX amended samples at the early and late time point, respectively. At 25 feet, only *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and unclassified *Enterobacteriaceae* were present as the ten most abundance phylotypes at both time points (Figure 6E, F). In general, the abundance of these phylotypes was low in the initial sample and the No RDX controls, indicating all benefited from RDX degradation.

For well 61, the most abundant phylotypes in the RDX amended microcosms also varied between the microcosms constructed from different depths and different time points (Figure 7). At day 45, common phylotypes (within the ten more abundant) during RDX degradation among the three depths included *Sporolactobacillus*, *Pseudomonas*, *Stenotrophomonas*, *Clostridium sensu stricto* and unclassified *Alcaligenaceae* (Figure 7A, C, E). In many cases, the abundance of

these phylotypes was low initially and in the No RDX controls, again indicating a benefit from RDX degradation. Notably, both *Sporolactobacillus* and *Pseudomonas* were the two most common phylotypes at all depths at day 45. At day 90, only *Methylophilus* was common across all three depths (Figure 7 B, D, F).

For well 61, comparing the common phylotypes across time, at 5 feet, only *Pedobacter* was common between the two time points (Figure 7A, B). At 10 and 20 feet, only *Pseudomonas* was common across the two time points (Figure 7C, D, E, F).

5.3.5. Phylotypes enriched during RDX degradation in groundwater microcosms (wells 1, 10, site 2)

Similar comparisons were made between the most common phylotypes in 1) the RDX amended groundwater microcosms, 2) the initial samples and 3) the No RDX control microcosms (Figure 8). For the downstream groundwater microcosms (well 1), three phylotypes (*Pseudomonas*, unclassified *Rhodocyclaceae* and *Sphingomonas*) were common in the RDX amended microcosms both with and without glucose (Figure 8A, B). However, these phylotypes were also relatively high in the initial samples and the No RDX control microcosms. In the upstream microcosms (well 10), only unclassified *Bacteroidetes* and *Pseudomonas* were common phylotypes when RDX degradation occurred (Figure 8C, D). In two of the four experiments *Rhodococcus* was among the ten most abundant phylotypes (Figure 8A, D).

5.4. Discussion and Conclusions

The potential for *in situ* biodegradation of any contaminant is difficult to predict, in part, because of the uncertainty associated with the characteristics and capabilities of the *in situ* microbial community. In the past decade, the use of molecular methods has helped to address this limitation. This study contributes to an improved understanding of RDX biodegradation potential through providing data on the phylotypes and genes linked to RDX removal in sediment and groundwater samples from two sites. For this, the most dominant microorganisms in microcosms containing sediment from different depths or groundwater from down- and upstream of an emulsified biobarrier during RDX degradation were identified. While it cannot be concluded that these microorganisms were growing on RDX, the results indicate the phylotypes benefited from the degradation process. Also, three functional genes associated with RDX

degradation (*xenA*, *xenB* and *xplA*) were quantified before and during RDX degradation. As all three genes have been associated with RDX degradation under oxygen depleted conditions, it was expected that gene copies of all three could increase in these microcosms. To our knowledge, the distribution of RDX functional genes throughout a sediment profile for a contaminated site has not previously been reported. Additionally, the literature does not contain such information for groundwater samples surrounding a biobarrier.

The current research indicates that a variety of phylotypes increased in abundance during RDX degradation. For the experiments involving sediments from well 58, *Pseudomonas*, *Rhodococcus* and *Arthrobacter* were particularly important during RDX degradation at all three depths examined. These data support the literature, as isolates of both *Pseudomonas* and *Rhodococcus* have been associated with RDX degradation in previous studies (Cupples 2013). In addition, for well 58 (both time points) *Yersinia* and *Dyella* were dominant in the RDX degrading samples at the shallow depth. To date, these phylotypes have yet to be linked to RDX degradation in pure cultures.

For well 61, common phylotypes during RDX degradation among the three depths included *Sporolactobacillus*, *Pseudomonas*, *Stenotrophomonas*, *Clostridium sensu stricto* and unclassified *Alcaligenaceae*. Notably, both *Sporolactobacillus* and *Pseudomonas* were the two most common phylotypes at all depths at day 45. At day 90, only *Methylophilus* was common across all three depths, however, it is likely this phylotype is consuming an RDX metabolite. Again, these data illustrate an important role for *Pseudomonas* in RDX biodegradation. A study on RDX degradation in groundwater samples from two navy sites (Pueblo Chemical Depot, CO and Picatinny Arsenal, NJ) has also detected *Clostridium* and *Sporolactobacillus* among the dominant 16S rRNA sequences (Fuller et al. 2010).

In the experiments involving downstream groundwater, three phylotypes (*Pseudomonas*, unclassified *Rhodocyclaceae* and *Sphingomonas*) were common during RDX biodegradation in microcosms both with and without glucose. In the upstream microcosms, both unclassified *Bacteroidetes* and *Pseudomonas* were common phylotypes when RDX degradation occurred. In addition, *Rhodococcus* was again noted as a dominant phylotype in a subset of these experiments following RDX degradation. The unclassified *Rhodocyclaceae* partial 16S rRNA gene sequence found here was 100 % similar to *Propionivibrio militaris* (NCBI reference sequence:

NR_125528.1), a perchlorate-reducing bacteria [36]. This result is not surprising as the site is co-contaminated with RDX and perchlorate.

Overall, the data indicate that the known RDX degrading genera, *Pseudomonas* and *Rhodococcus*, are associated with RDX degradation in these experiments. Further, the results suggest an important role for genera that do not contain isolates previously linked to RDX degradation including *Arthrobacter*, *Yesinia*, *Dyella*, *Sporolactobacillus*, *Stenotrophomonas* and *Sphingomonas*.

The overall aim of this research was to correlate functional gene and phylotype data during RDX biodegradation. For the experiments involving sediment samples from well 58, significant increases were observed for *xplA*, *xenA* and *xenB* at all three depths (primarily at day 130). Correspondingly, at day 130, both *Rhodococcus* (*xplA*) and *Pseudomonas* (*xenA*, *xenB*) were abundant at depth 25 feet. However, at 5 feet (day 130), *Pseudomonas* was abundant, but *Rhodococcus* was not. Whereas, at 10 feet, *Rhodococcus* was abundant, but *Pseudomonas* was not. It is possible that other microorganisms were associated with these functional genes in these microcosms.

For well 61, *xplA* increased in abundance at all depths at both days 45 and 90. However, *Rhodococcus* was only highly abundant in two of the six experiments. For well 61, gene copy numbers for *xenA* were higher only for 10 feet deep microcosms at both time points and *Pseudomonas* was one of the ten most abundant phylotypes in these microcosms. Also for well 61, gene copy numbers for *xenB* was higher only for 20 feet deep microcosms and again *Pseudomonas* was important in these samples.

Similar results were obtained for linking function with identity for the groundwater samples. Gene number of *xplA* increased in well 1 microcosms (without glucose) and *Rhodococcus* was also abundant in these samples. Gene copies of *xenA* and *xenB* also increased in well 1 microcosms and again *Pseudomonas* was important in these microcosms. In contrast, in well 10 microcosms (upstream of the biobarrier), no significant change was observed in *xplA*, even though *Rhodococcus* was present in the samples amended with glucose and RDX. A significant change was noted for *xenB* (not for *xenA*) and, correspondingly, *Pseudomonas* was abundant in the samples from this well. The functional gene and phylotype data collected here suggest that the enzymes encoded by *xplA*, *xenA* and *xenB* are all important for RDX biodegradation in these samples. Further, in many cases, the phylotypes previously associated

with these genes were enriched during RDX degradation.

From an applied perspective, the data collected here indicate the emulsified biobarrier was beneficial for RDX bioremediation. Evidence for this includes the more rapid RDX removal trend in microcosms from well 1 compared to well 10. In addition, a greater increase in functional genes occurred in the microcosms inoculated with well 1 compared to well 10 groundwater. Further *Pseudomonas*, a likely key RDX degrader, was a significant portion of the microbial community in samples from well 1.

The ability to monitor specific microorganisms and/or genes coding enzymes involved in RDX degradation in environmental samples is important for remediation strategies that rely on biological removal. Favoring this goal, primers were developed towards *xenA*, *xenB* and *xplA* and the importance of each gene during RDX degradation was determined. These primers should be advantageous for investigating the feasibility of bioremediation across RDX contaminated sites. Future research focusing on additional functional genes associated with RDX degradation (e.g. *diaA*, *hydA*) would be beneficial. Further, the role of the novel genera identified here associated with RDX degradation, such as *Sporolactobacillus* and *Arthrobacter*, should be explored.

Gene and Sample	Range	Slope (Average \pm St dev)	R ² (Average \pm St dev)	Amplification Efficiency (%) (Average \pm St dev)
<i>xplA</i> well 58	10 ³ -10 ¹⁰	-3.18 \pm 0.09	0.97 \pm 0.01	106.3 \pm 4.2
<i>xenA</i> well 58	10 ³ -10 ⁹	-3.51 \pm 0.05	0.97 \pm 0.02	92.8 \pm 1.7
<i>xenB</i> well 58	10 ³ -10 ¹⁰	-3.59 \pm 0.02	0.99 \pm 0.002	90.1 \pm 0.6
<i>xplA</i> well 61	10 ³ -10 ¹⁰	-3.18 \pm 0.08	0.96 \pm 0.01	106.3 \pm 3.5
<i>xenA</i> well 61	10 ³ -10 ⁹	-3.34 \pm 0.46	0.96 \pm 0.02	101.7 \pm 20.9
<i>xenB</i> well 61	10 ³ -10 ¹⁰	-3.99 \pm 0.39	0.99 \pm 0.01	78.7 \pm 10.6
<i>xplA</i> well 1, 10	10 ³ -10 ¹⁰	-3.51 \pm 0.33	0.95 \pm 0.02	93.9 \pm 12.9
<i>xenA</i> well 1, 10	10 ³ -10 ¹⁰	-3.18 \pm 0.02	0.98 \pm 0.02	106.3 \pm 0.9
<i>xenB</i> well 1, 10	10 ³ -10 ¹⁰	-2.83 \pm 0.19	0.97 \pm 0.01	125.9 \pm 12.9

Table 5.1. Sequence, target and amplicon length of the primers designed to amplify the three RDX-degrading functional genes.

Table 5. 2. Characteristics of the standard curves used to enumerate *xplA*, *xenA* and *xenB* in sediment (wells 58 and 61, site 1) and groundwater (wells 1 and 10, site 2) microcosms.

Table 5.3. Summary MiSEQ Illumina data generated in this study. Data are shown for microcosms derived from RDX-contaminated sediments and controls from well 58 after 90 and 130 days of incubation and well 61 after 45 and 90 days of incubation. Data are also shown from microcosms of groundwater collected from the upstream and downstream of a biobarrier.

Target Gene	Forward or Reverse	Sequence (5' to 3')	Tm (°C)	Gene Target Match (Accession #)	Target Match Position	Target Size (bp)
<i>xplA</i>	Forward	AGGCTATCGCCACGATTCTG	59.97	<i>Rhodococcus rhodochrous</i> strain 11Y cytochrome P450-like protein XplA (AF449421)	1139 to 1158	382
<i>xplA</i>	Reverse	ATCTGTCCCGCACAGGAATG	60.11	As above	1520 to 1501	382
<i>xenA</i>	Forward	CACCATTCCTCGAGACCAACA	59.96	<i>Pseudomonas putida</i> xenobiotic reductase A (AF154061)	909 to 928	324
<i>xenA</i>	Reverse	TTTAGATTCTGGGGGCTGCTG	60.11	As above	1232 to 1213	324
<i>xenB</i>	Forward	ACCTTCACCTATGTTGCTCGC	60.68	<i>Pseudomonas fluorescens</i> xenobiotic reductase B (AF154062)	931 to 951	351
<i>xenB</i>	Reverse	CGTTTCTAGCGTTTCATGCGGT	62.05	As above	1281 to 1260	351
Total MiSEQ/Illumina data vs. time point		Sequences Following Make Contigs	Unique Sequences	Final Sequences	% Chimeric	

Well 58: 5, 10, 20 ft, day 90 (RDX)	1,202,168	107,593	688,088	9.04
Well 58: 25, 30 ft, day 90 (RDX)	1,006,806	92,868	587,112	9.78
Well 58: 5, 10, 20 ft, day 90 (No RDX)	1,528,362	128,184	861,211	11.17
Well 58: 25, 30 ft, day 90 (No RDX)	1,166,525	97,644	678,201	12.16
Well 58: 5 ft day 130 (RDX, No RDX)	766,530	95,970	525,534	15.83
Well 58: 10 ft day 130 (RDX, No RDX)	710,730	16,106	452,224	7.83
Well 58: 25 ft day 130 (RDX, No RDX)	512,091	50,554	337,668	5.45
Well 61: 5, 10, 20 ft, day 45 (RDX)	1,664,542	175,060	1,000,936	14.01
Well 61: 5, 10, 20 ft, day 45 (No RDX)	1,535,720	147,269	904,554	11.84
Well 61: 5, 10, 20 ft, day 90 (RDX)	1,397,018	183,178	763,446	11.22
Well 61: 5, 10, 20 ft, day 90 (No RDX)	959,894	58,737	419,640	1.21
Well 1 (RDX, No RDX)	1,298,780	116,268	836,404	13.37
Well 1 + glucose (glucose+RDX, glucose)	889,728	80,769	556,922	15.05
Well 10 (RDX, No RDX)	926,087	80,650	544,862	6.64
Well 10 + glucose (glucose+RDX, glucose)	1,002,615	86,236	647,829	13.97

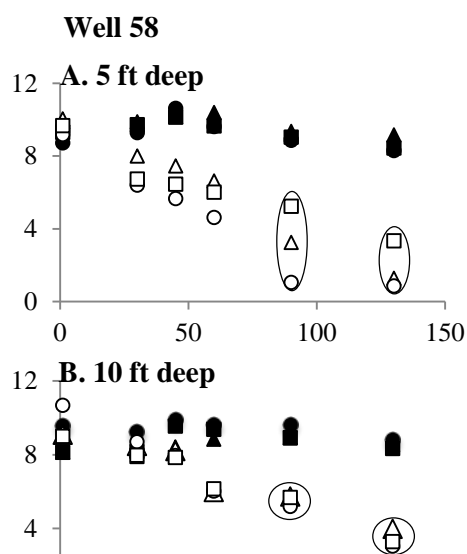
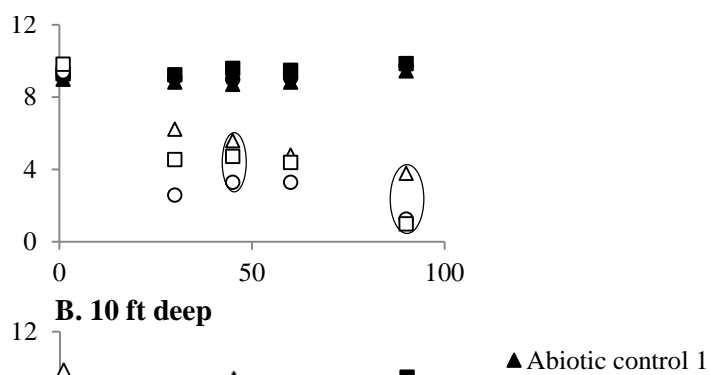




Figure 5.1. RDX concentrations (mg/L) in RDX amended microcosms and abiotic controls containing sediment from well 58 at 5 ft deep (A), 10 ft deep (B), 20 ft deep (C), 25 ft deep (D) and 30 ft deep (E). The samples subject to DNA extraction and community/functional gene analysis are circled (days 90 and 130).



RDX concentration (mg/L)

Figure 5.2. RDX concentrations (mg/L) in RDX amended microcosms and abiotic controls containing sediment from well 61 at 5 ft deep (A), 10 ft deep (B) and 20 ft deep (C). The samples subject to DNA extraction and community/functional gene analysis are circled (days 45 and 90).

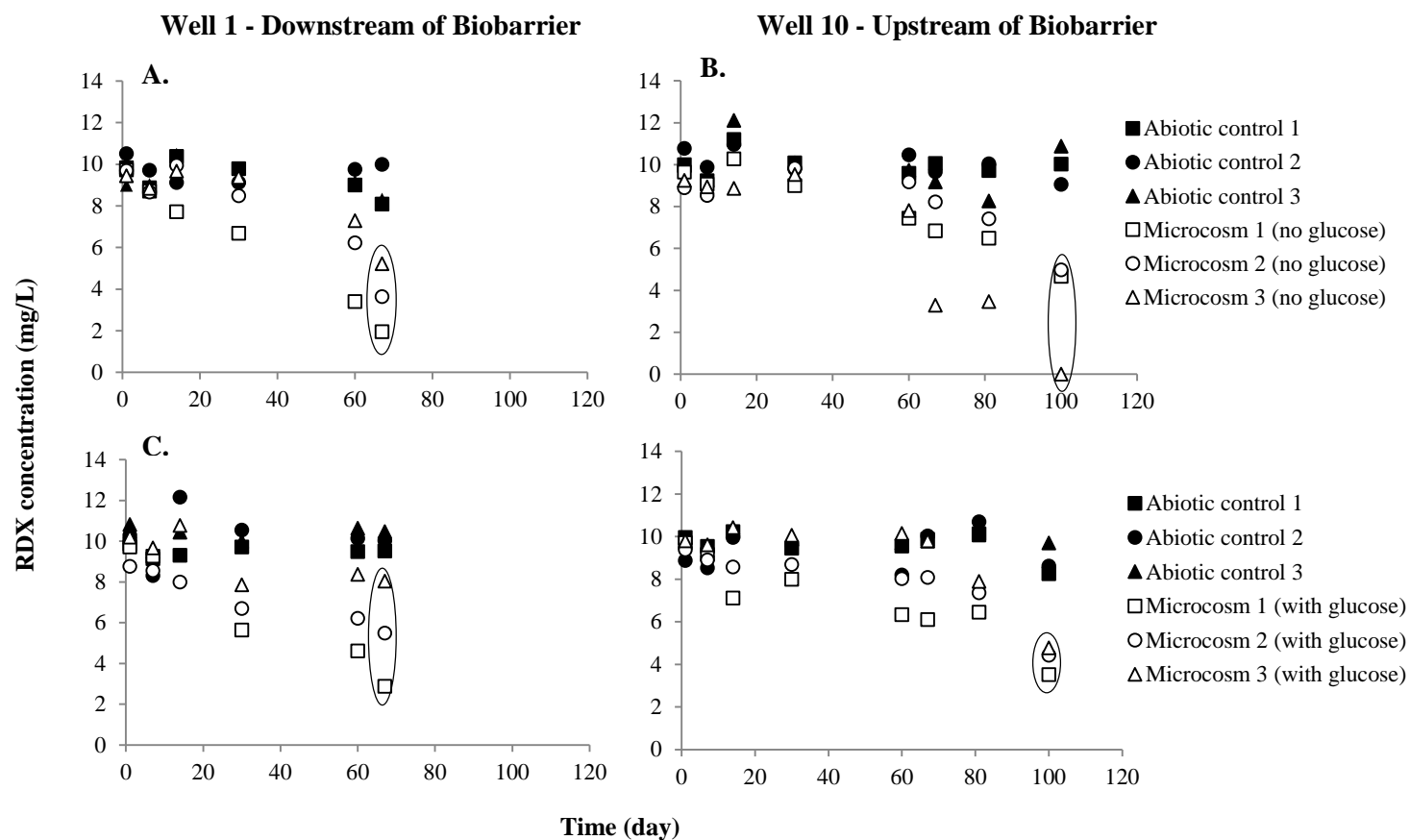


Figure 5.3. RDX concentrations (mg/L) in RDX amended microcosms and abiotic controls containing groundwater from downstream (A, C) and upstream (B, D) of the buffered emulsified oil biobarrier. The samples subject to DNA extraction and community/functional gene analysis are circled (day 67 and day 100 for upstream and downstream, respectively).

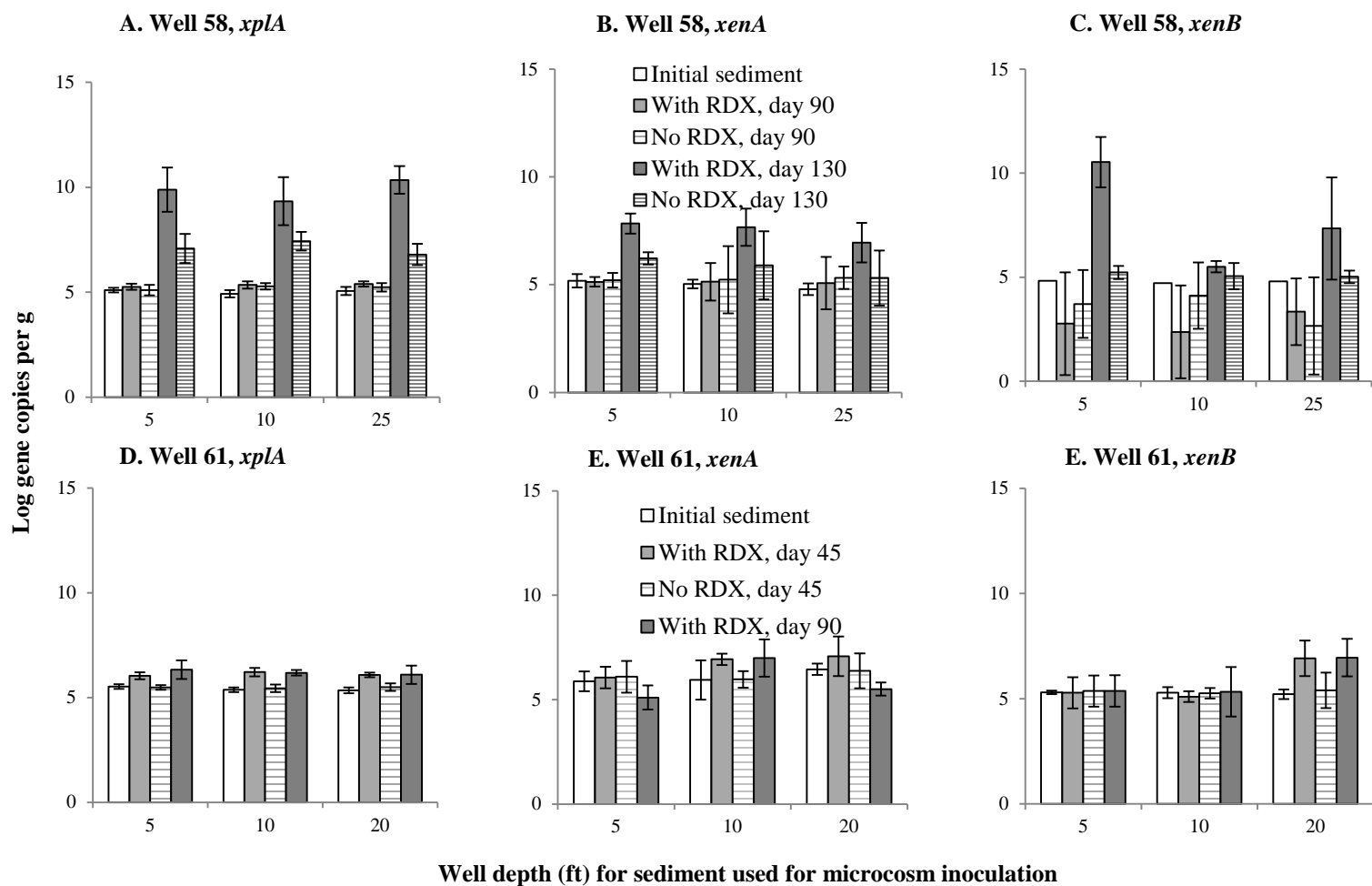


Figure 5.4. Average (\pm standard deviations, $n=3$) log gene copies of *xplA*, *xenA* and *xenB* in the initial sediments and in microcosms derived from sediment from well 58 (A, B, C) and well 61 (D, E, F). Values are shown for RDX amended microcosms and for no RDX controls at different DNA extraction times. The microcosms were constructed with sediment from different depths at these wells (5, 10 and 25 ft for well 58 and 5, 10 and 25 ft for well 61).

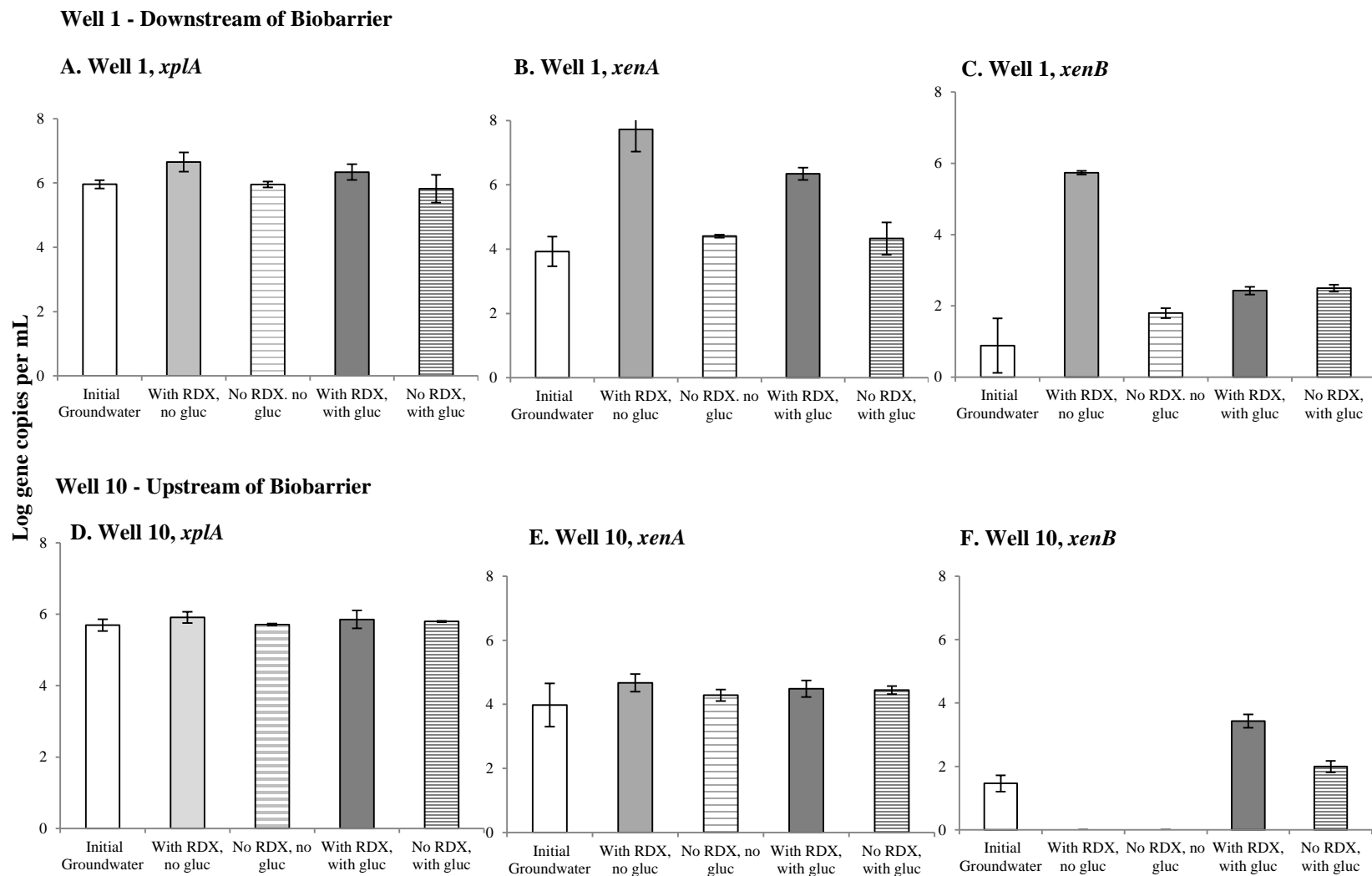


Figure 5.5. Average (\pm standard deviations, $n=3$) log gene copies of *xplA*, *xenA* and *xenB* in the initial groundwater and in microcosms derived from that groundwater from well 1 (downstream of biobarrier, A, B, C) and well 10 (upstream of biobarrier, D, E, F). Values are shown for RDX amended microcosms and for no RDX controls, both with and without glucose.

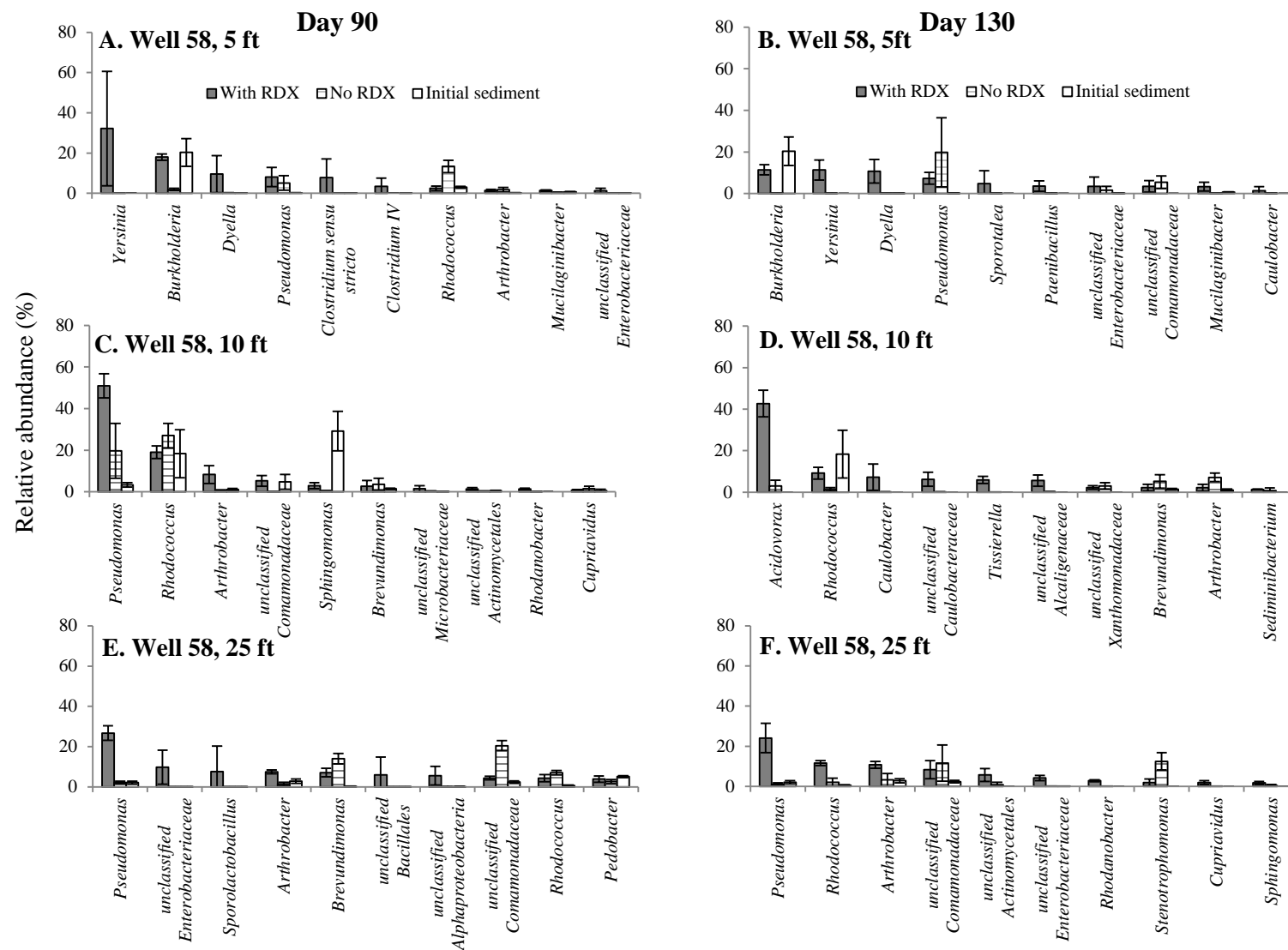


Figure 5.6. Relative abundance (%) of the ten most abundant phylotypes in RDX amended microcosms compared to the no RDX control microcosms and the initial sediment from well 58. The microcosms were inoculated with sediment (well 58 from depths 5 ft (A, B), 10 ft (C, D) and 25 ft (E, F) and DNA was extracted at days 90 (A, C, E) and 130 (B, D, F). Values represent averages and the bars represent standard deviations from three samples.

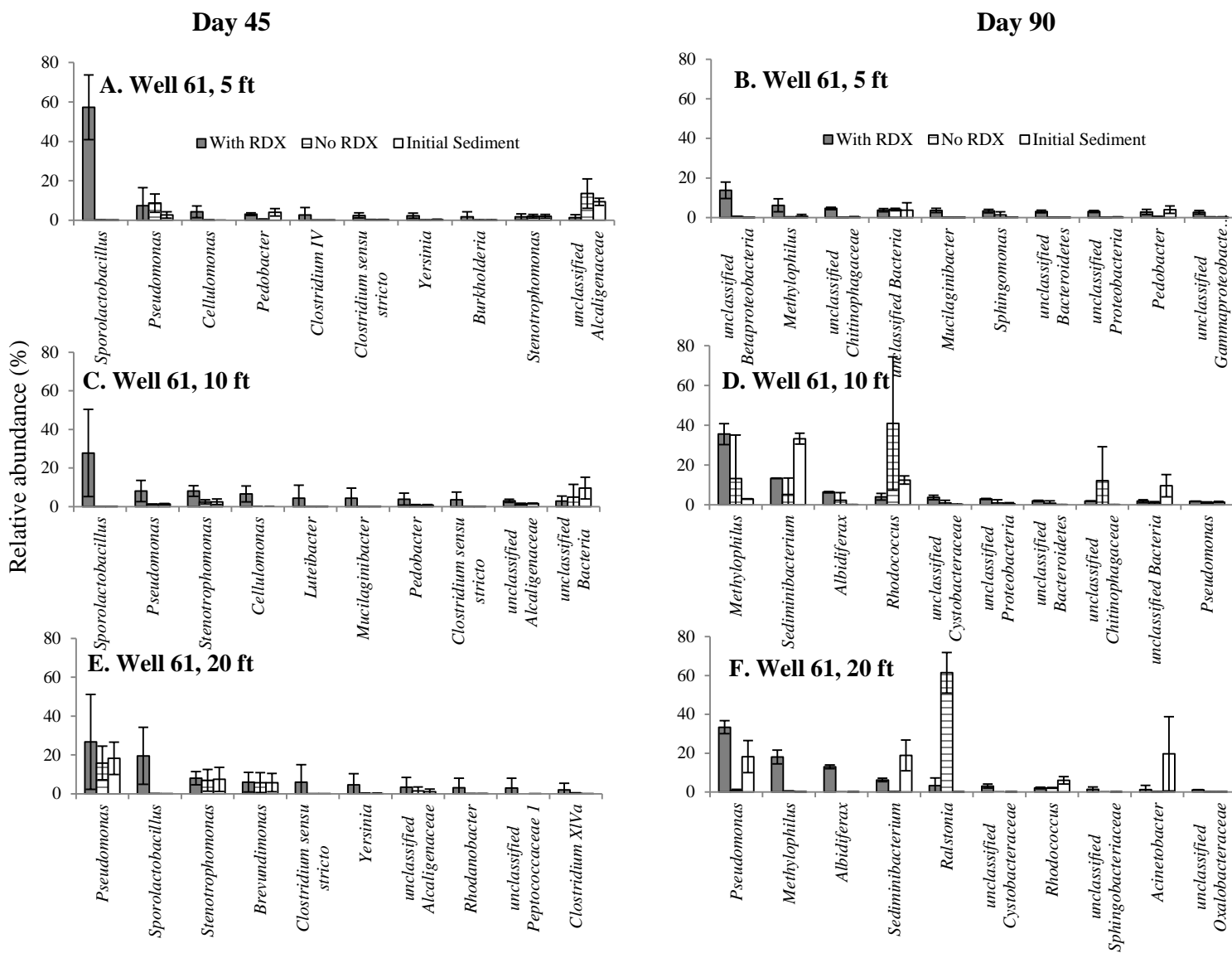


Figure 5.7. Relative abundance (%) of the ten most abundant phylotypes in RDX amended microcosms compared to the no RDX control microcosms and the initial sediment from well 58. The microcosms were inoculated with sediment (well 61 from depths 5 ft (A, B), 10 ft (C, D) and 25 ft (E, F) and DNA was extracted at days 45(A, C, E) and 90 (B, D, F). Values represent averages and the bars represent standard deviations from three samples.

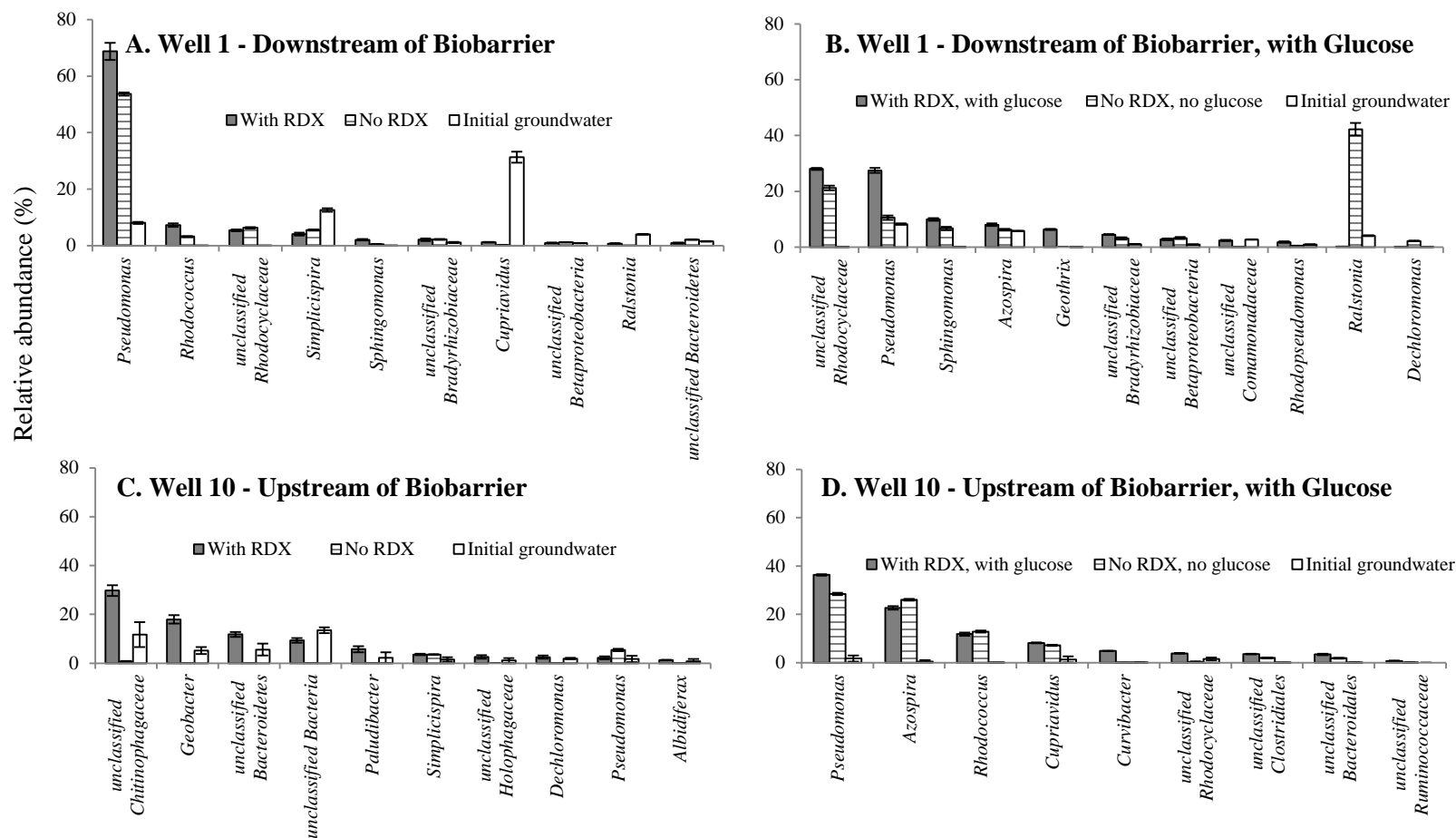


Figure 5.8. Relative abundance (%) of the ten most abundant phylotypes in RDX amended microcosms compared to the no RDX control microcosms and the initial groundwater from wells 1 (A, B) and 10 (C, D). A number of microcosms were also amended with glucose (A, D). Values represent averages and the bars represent standard deviations from three samples.

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APPENDICES

Peer Reviewed Manuscripts

- 1 Paes Wilson F., Cupples A.M. (in press) Microbial community characterization and functional gene quantification in RDX degrading microcosms derived from sediment and groundwater at two Naval sites. *Applied Microbiology and Biotechnology*.
- 2 Jayamani I., Cupples A.M. (2015b) Stable isotope probing reveals the importance of *Comamonas* and *Pseudomonadaceae* in RDX degradation in samples from a Navy detonation site. *Environmental Science and Pollution Research* 22(13):10340-10350 doi:10.1007/s11356-015-4256-6
- 3 Jayamani I., Cupples A.M. (2015) A comparative study of microbial communities in four soil slurries capable of RDX degradation using Illumina sequencing. *Biodegradation* 26(3):247-257 doi:10.1007/s10532-015-9731-8.
- 4 Cupples A.M. 2013. RDX degrading microbial communities and the prediction of microorganisms responsible for RDX bioremediation. *International Biodeterioration and Biodegradation* 85:260-270.

Presentations

- 1 Cupples, A. M. and F. Paes. 2016. Linking phylogeny and function in RDX degrading communities from groundwater and sediment from two Naval sites. The Tenth International Conference on Remediation of the Chlorinated Solvents and Recalcitrant Compounds. Palm Springs, 25th May 2016
- 2 Cupples, A. M., I. Jayamani, F. Paes. 2015. Development of biomarkers for assessing *in situ* RDX biodegradation potential. Strategic Workshop on Research and Demonstration Needs for Management of Munition Constituents. July 28-29th, Washington Navy Yard, DC.
- 3 Cupples, A. M. and Indumathy Jayamani. 2015. Novel microorganisms linked to RDX degradation across soil communities. Third International Symposium on Bioremediation & Sustainable Environmental Technologies, Miami, FL. May 20th 2015.
- 4 Jayamani, I. and A. M. Cupples. 2014. A comparison of RDX assimilating microorganisms across soil communities. May 21st, 2014. Ninth International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey, CA, May 19-22, 2014.
- 5 Cupples, A. M. and I. Jayamani. 2014. Development of biomarkers for assessing *in situ* RDX biodegradation potential. SERDP In Progress Review Meeting, Arlington, VA, May 6th 2014.
- 6 Jayamani, I. and A. M. Cupples. 2014. A comparison of RDX assimilating microorganisms across soil communities. Engineering Graduate Research Symposium, March 24th, 2014, Breslin Center.
- 7 Cupples, A. M. 2013. ER-1609: Development of biomarkers for assessing *In Situ* RDX biodegradation potential. SETAC/ESTCP Progress Review Meeting, Washington DC.
- 8 Paes, F., I. Jayamani, A. M. Cupples. 2012. Stable isotope probing to identify the microorganisms able to degrade the explosive RDX. Engineering Graduate Research Symposium, November 9th, 2012, Breslin Center.
- 9 Cupples, A. M. 2010. ER-1609: Development of biomarkers for assessing *In Situ* RDX biodegradation potential. SETAC/ESTCP Progress Review Meeting, Washington DC.